Molecular Analysis of *Schistosoma japonicum* Phosphatidylinositol Glycan – Class N gene

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A Thesis Submitted in Partial Fulfilment
Of the Requirements for the Degree of
Master of Philosophy
In
Biology

September 2003

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Statement

All the experimental work reported in this thesis was performed by the author, unless otherwise specified.

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Acknowledgements

I am greatly indebted to my supervisor, Dr. M.C. Fung, for providing me the opportunity to study in the field of the biology of *Schistosoma japonicum*, which is of particular interest and a challenging task to me. I am grateful for his skillful and patience guidance, providing much suggestions and comments for improvements, and also encouragement and support, which direct me to the completion of the project. I would also like to express my sincere gratitude to the members of the thesis committee, Dr. W. Ge and Dr. V.E.C. Ooi, and external examiner, Dr. N.K. Mak for their patience of reviewing my thesis and invaluable comments.

I would like to thank Dr. N.K. Mak at the Hong Kong Baptist University for contribution to the experiment of Sj-PIG-N protein localization. I am particular grateful to Dr. T. Kinoshita at Osaka University for providing F9 mouse cell line and PIG-N double knockout F9 mouse cell line, and his contribution to the biochemical function test of Sj-PIG-N gene. I would like to acknowledge Dr. Z.D. Wu at Sun Yat – Sen University of Medical Sciences for providing *S. japonicum* infected serum and *S. japonicum* cercaria. I also thank Dr. X.G. Chen for the construction of S. japonicum cercaria cDNA library and his valuable opinion and discussion.

I should appreciate Mr. Freddie, C.H. Kwok, and Mr. Wilson, K.W. Lau for their expert and enthusiastic technical assistance and helpful advice. My appreciation also go to Miss Pandora, M. C. Yip for her excellent management laboratory and understanding. I would like to thank my labmates, Miss. Polly, S. M. Chan, Mr. Jacky, C.S. Lo, Mr. Kit, S.K. Lo, Miss. Candy, W. Y. Ng, Miss. S.F. Sia, Miss. Terisa, P.C.

Leung, Mr. Lawrence, S.L. Tsang, for their help and sharing with me innumerable happy moments.

Last but not the least, I wish to express my thankfulness to my family for their unconditional love and support throughout the years which mean so much to me.

Abstract

Apart from the transmembrane proteins, many cell-surface proteins are linked to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor. GPI anchored proteins serve many important cellular functions. In mammalian cells, GPI anchored proteins contribute for the formation of microdomains. And, the pathway of GPI synthesis plays a role in cellular signaling. GPI anchored proteins are also very important in immune evading mechanism of parasites. For example, periodical changing of GPI anchored proteins - variant surface glycoproteins protects trypanosomes from the specific immune response of their host. In schistosome, the surface membranes of tegument layer bind a wide range of host molecules, which including the glycolipid moiety of the blood cell substances and proteins, such as the histocompatibility antigens and immunoglobulins, by specific capture mechanisms involving lipid carrier macromolecules, such as GPI anchored low density lipoprotein (LDL) receptors. GPI anchored proteins also are involved glucose uptaking in the parasite.

The synthesis pathway of the GPI anchor is complex, highly conserved in all the eukaryotic cells. For different organisms, they share a same main core structure of GPI anchor, EtN-P-Man-Man-Man-GlcN-PI. The synthesis pathways of GPI anchors in mammalian cells and yeast are well studied. However, no enzyme involved in GPI anchor synthesis has been reported in schistosome.

In screening of *S. japonicum* cercaria cDNA library, I have cloned a homologous of phosphatidylinositol glycan, class N (PIG-N), and named as Sj-PIG-N that encoded

987 amino acids. phosphatidylinositol glycan, class N is an enzyme that transfer ethanolamine phosphate (EtN-P) to the first mannose of the GPI more core structure in the GPI anchor synthesis. We also demonstrated that the Sj-PIG-N gene had a similar function of mouse PIG-N gene and localized the gene product in endoplasmic reticulum (ER) lumen membrane. Sj-PIG-N gene is the first gene involved in GPI anchor synthesis cloned in parasite schistosome. In trypanosome, enzymes in GPI anchor synthesis are the targets of anti-parasitic drug screening. Therefore, the identification of Sj-PIG-N provides a new direction for anti-schistosome drug screening.

摘 要

除了跨膜蛋白以外， 許多細胞表面蛋白通過糖基磷脂酰肌醇錨連接在原生質膜上。 糖基磷脂酰肌醇錨着點蛋白在細胞內扮演重要角色。 在哺乳动物細胞中， 糖基磷脂酰肌醇錨着點蛋白組成細胞細小區域。 而且糖基磷脂酰肌醇錨的合成路徑也具有細胞的信號傳遞功能。 糖基磷脂酰肌醇錨着點蛋白對於寄生蟲的免疫逃避機制也是非常重要。 例如錐蟲， 糖基磷脂酰肌醇錨着點蛋白 – 变異表面糖蛋白(VSG)的周期性改变能够有效的保护寄生蟲不受寄主免疫系统的傷害。 血吸蟲的皮層外膜連接着許多寄主的分子， 包括血細胞物質和蛋白的糖脂类部分， 例如組织适合性抗原和免疫球蛋白。 寄主的分子的捕捉是通過一類脂类載體大分子， 例如糖基磷脂酰肌醇錨着點低密度脂蛋白受體。 在血吸蟲中，糖基磷脂酰肌醇錨着點蛋白還作參與對寄主血液中葡萄糖的吸收。

在所有真核細胞中， 糖基磷脂酰肌醇錨的合成途徑是複雜和高度保守。 糖基磷脂酰肌醇錨的核心結构在不同的生物體中都是一样的。 該核心結构就是 EtN-P-Man-Man-Man-GlcN-PI。 雖然糖基磷脂酰肌醇錨的合成在哺乳动物細胞和酵母菌中已經研究得十分深入了， 但是在血吸蟲中的研究卻非常有限。 至今爲止， 尚未有糖基磷脂酰肌醇錨合成酶在血吸蟲中被報導過。

在篩選日本血吸蟲(*Schistosoma japonicum*)尾蚴互補去氧核糖核酸(cDNA)文庫時， 本人克隆了一個老鼠的磷脂酰肌醇聚糖分类N的同源基因， Sj-PIG-N。該基因編碼有 987 個氨基酸。 在糖基磷脂酰肌醇錨的合成過程中， 磷脂酰肌醇聚糖分类N的功能是傳送一個乙醇胺磷酸分子(EtN-P)到糖基磷脂酰肌醇錨核心結构的第一個甘露糖上。 我們不但証明了 Sj-PIG-N 有着同老鼠的磷脂酰肌醇聚糖分类N相同的功能， 而且确定 Sj-PIG-N 作用於細胞內質网的內腔膜上。

Sj-PIG-N 是第一個在血吸蟲中發現的糖基磷脂酰肌醇錨合成酶。 因爲在對抗錐蟲的研究中， 糖基磷脂酰肌醇錨合成酶是篩選抗寄生蟲药物的主要目標之一，並取得了一定成果。 所以 Sj-PIG-N 的發現爲研制抗血吸蟲药提供了一個新的方向。

Abbreviation

Abbreviation used in the thesis without definition include:

AChE	Acetylcholinesterase
Amp100	Ampicillin, 100μg/ml
cDNA	Complementary DNA
CTAB	Cetyldimethylethylammonium bromide
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates (dATP, dTTP, dGTP, dCTP)
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
ER	endoplasmic reticulum
EtN-P	ethanolamine phosphate
EtOH	Ethanol
GPI	glycosylphosphatidylinositol
IPTG	Isopropyl-β-D-thio-galactopyranoside
Kan 50	Kanamycin, 50μg/ml
kb	Kilo-base pairs
kDa	Kilo-dalton
M-MLV	Molony murine leukemia virus
MOPS	Morpholinopropanesulfonic acid
mRNA	Messenger RNA
O.D.	Optical density
PBS	Phosphate buffered saline, pH 7.4

PCR	Polymerase Chain Reaction
PIG-N	phosphatidylinositol glycan, class N
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
TAE	Tris-acetate-EDTA
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultraviolet
v/v	volume by volume
w/v	weight by volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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1.1 The Genus Schistosoma

Genus *Schistosoma* belongs to the family Schistosomatidae. Members of the family Schistosomatidae are dioecious Digenea, parasitic in the blood-vascular system of vertebrates. A common feature of the family is that the mature females is more slender than the male and carried in the gynaecophoric canal- a ventral groove formed by ventrally flexed lateral outgrowths of the male body. The genus *Schistosoma* is the only one in the family that is associated with mammals. In the mammalian blood flukes, the genus *Schistosoma* achieved the greatest geographical distribution and diversificationin the terms of numbers of recognized species and different host parasitized. Until now, there are eighteen species identified in Schistosoma. (Table1.1)

Table 1. Details of Schistosoma species, including the snail host, distribution and definitive mammalian host. (Rollinson and Simpson, 1987)

Schsitosoma species	Intermediate host genus	Definitive Host	Continental distribution
S. haematobium	*Bulinus senegalensis*	Primates	Africa
S. intercalatum	*Bulinus senegalensis*	Primates	Africa
S. mattheei	*Bulinus senegalensis*	Artiodactyla Primates	Africa
S. bovis	*Bulinus senegalensis* *Planorbarius metidjensis*	Artiodactyla	Africa

Schsitosoma species	**Snail host genus**	**Host**	**Continental distribution**
S. curassoni	*Bulinus senegalensis*	Artiodactyla	Africa
S. margrebowiei	*Bulinus senegalensis*	Artiodactyla	Africa
S. leiperi	*Bulinus senegalensis*	Artiodactyla	Africa
S. Mansoni	*Biomphalaria pfeifferi*	Primates Rodentia	South America, Africa Caribbean, Madagascar
S. rodhaini	*Biomphalaria pfeifferi*	Rodentia Carnivora	Africa
S. edwardiense	*Biomphalaria pfeifferi*	Artiodactyla	Africa
S. hippopotami	*?*	Artiodactyla	Africa
S. indicum	*Indoplanorbis exustus*	Artiodactyla	India, Sri Lanka Southeast Asia
S. spindale	*Indoplanorbis exustus*	Artiodactyla	India, Southeast Asia
S. nasale	*Indoplanorbis exustus*	Artiodactyla	India, Sri Lanka
S. incognitum	*Lymnaea luteola* *Radix rubiginosa*	Rodentia, Carnivor, Artiodactyla	India Southeast Asia
S. japonicum	*Oncomelania hupensis*	Primates, Rodentia, Carnivora, Perissodactyla, Artiodactyla	China Japan Phillipines Inodesia
S. mekongi	*Tricula aperata*	Primates, Carnivora	Southeast Asia
S. sinensium	*Tricula aperata*	Rodentia	Southeast Asia

1.2 Biology of *Schistosoma japonicum*

1.2.1 The History of discovery of *Schitosoma japonicum*

Schistosomiasis japonica is an infectious disease caused by the Asian species of trematodal parasite *Schistosoma japonicum*. The earliest record of the disease - Schistosomiasis japonica was from Fujii in katayama district, 1847 (Fujii, 1847). Later, the symptoms of the disease were listed by professor Baelz, the Imperial University, Yokyo, 1883. However, the disease was misinterpreted as being related to the Okayama infection caused by Chlonorchis sinensis. Until 1904, the *S. japonicum* was identified and related to the disease by Katsurada, who gave an accurate description of the *S. japonicum* male and female parent worms, the eggs and the pathological picture of the disease (Katsurada, 1904; Katsurada, 1904a).

1.2.2 Life cycle of *Schistosoma japonicum*

The life cycle of *S. japonicum* is complex and encompass development in tow hosts and short-lived water-born forms (reviewed by *Ross et al.,* 2001). In its adult worm stage, the parasite is an intestinal worm that lives in the mesenteric veins outside of its definitive host liver. In China, there are at least 37 vertebrate hosts have been identified for *S. japonicum.* Inside the definitive host, male and female worms paired for reproduction. Worm eggs pass through the membranes of the bowels and are excreted in stool. After leaving the host, eggs hatch into free-swimming larva when they contact with water environment. The free-swimming larva is called miracidia. Miracidia can infect the secondary host – *Oncomelania hupensis* within its

lifespan. Once the intermediate larva forms has infected a snail, they initiate the asexually reproduction through a series of stages called sporocyts. Finally, sporocyts can develop into another free-swimming from larva, cercaria. During the asexual reproduction stage, single miracidia infection can produce several thousand cercariae over the lifetime of the infected snail. The cercariae development period in snail is strongly dependent on environmental temperature and varies between seven weeks in summer and ten weeks in winter. After completing the development, cercariae migrate to the surface of snail and are 'shed' into environment. Free-swimming cercariae have to infect a suitable vertebrate host to continue their development. The cerceriae are attracted to skin oil and other chemicals given off by host body. When they come into contact with a host's skin, they can release proteases to penetrate the skin layer of host. During the skin penetration, cercaria shed its tail in the process and becomes schistosomula. Once the parasite enters host body, a circuitous migration through the host body leads the maturing schistosomula larva to its final destination near the liver. At last, Schistosomulas mature into adult worms and reproduce in the definitive host. A new cycle starts. The (Fig. 1) presents the entire life cycle of *Schistosma japonicum*.

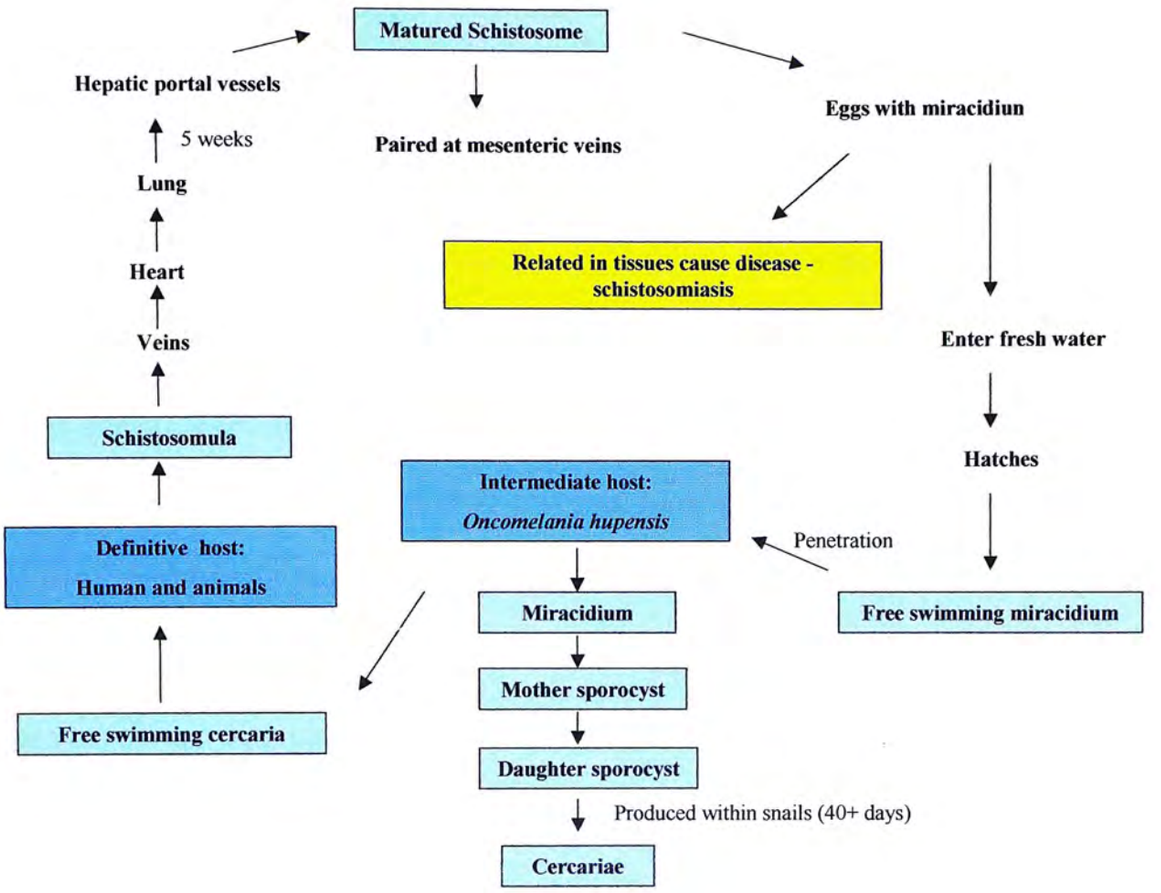


Fig. 1. Life cycle of *Schistosoma japonicum*.

1.2.2.1 Egg

The fertilized egg of *S. japonicum* is formed in the uterus of the adult female worm and deposited in the wall of gut of host animal. The fertilized eggs finally leave host body with feces. However, eggs found in uterus of female and that found in feces are definitively different in size and shape. Fertilized egg, in uterus, is a lenticular, ovoid object, about 67 to 50μ in size, and bears a pronounced spine on the side near the anterior end. The egg found in feces is an oval object with an average 80μ in diameter, and 66μ in breath (Braun Seifert, 1915; Fantham et al., 1916; Castellani and Chalmers, 1919; Manson-Bahr, 1921). The migration of eggs from blood vessel to feces is a very complex process. There are numerous interacting factors involved (Smith, 1974; Bloch, 1980): the spine of the egg, blood vessel, peristalsis, and proteolytic enzymes secreted by miracidia. So far, no study of circadian rhythm of egg excretion to outside environment that have been reported for *S. japonicum*. Based on the studies in *S. haematibium* (MacMahon, 1976; Pugh, 1979), the rhythms of egg excretion is synchronized by nycthemeral variations of body temperature. In *S. japonicum*, each pair of worms excretes 66-495 eggs per day (Loker, 1983). Moreover, the viability of *S. japonicum* eggs is very high. The egg can remain viable for at least a week outside the host body (Garcia, 1976).

1.2.2.2 Miracidia

When egg is released outside the host accompanied with feces, it usually contains a mature or nearly mature miracidium. The hatching of schistosome eggs in the outside environment only occurs in suitable environmental condition including external and internal factors. One of the internal factors is the maturity of the

miracidium. The optimum temperature for the egg hatching is between 25°C to 30°C (Miyagwa, 1916). During the hatching, the shell membrane of egg splits along the line of least resistance that allows the shell to retract and miracidium to escape. The first 60-72 hours of miracidium is called infective period. Once the miracidium comes to the intermediate host – snail, it attacks the snail. The penetration points are found over the whole of the cephalopedal area (Jourdane and Xia, 1987).

Typically, miracidium is 78 to 120μ in length by 30 to 40μ in breath. Being a free-swimming organism, the mature miracidium has epidermal cillia for the adaptation of living environment.

1.2.2.3 Sporocysts

After penetrating the snail, miracidium can migrate towards the location where it can develop. For different species, the development locations are different. Miracidium of *S. japonicum* can invade all the tissue of snail, with a preference for the cavital organs, such as heart, visceral cavities (Jourdane and Xia, 1987). With fine morphological changes, miracidium develops into the mother sporocyste within several days. In *S. japonicum*, the mother sporocyst has a long sac shape. After that, the mother sporocyst differentiates into daughter sporocysts. The differentiation process includes two phases: the first phase is the multiplication of germinal cells in great numbers; the second phase is the individualization of daughter cells (Schutte 1974). Newly generated daughter cells leave the mother sporocyte and migrate to the digestive gland in the snail (Pan, 1965; Becker, 1970; Cheng and Bier, 1972; Meuleman, 1972; Schutte, 1974). The daughter sporocysts look like vermiform larvae, with 250μm x 40μm in size (Faust and Meleney, 1924). When they arrived the

location, they develop and increase greatly in size, and finally differentiate into cercarial embryo. However, the production of daughter sporocysts is not only direct to the cercarial stage, but also to the new daughter sporocysts production (Jourdane and Theron, 1980; Jourdane et al., 1981, 1984, 1985). Until now, there are three process of sporocystogenesis have been discovered.

(a) Direct sporocytogenesis. Daughter sporocysts generated from mother sporocysts produce new sporocysts directly.

(b) Sporocystogenesis after cercariogenesis. The daughter sporocysts start to produce new sporocysts only after their cercariae production stopping (Theron and Jourdane, 1979).

(c) Simultaneous sporocystogenesis and cercariogenesis. The daughter sporocyst produces cercariae and new sporocysts in same time (Theron, 1981; Combes, 1982; Fournier and Theron, 1985).

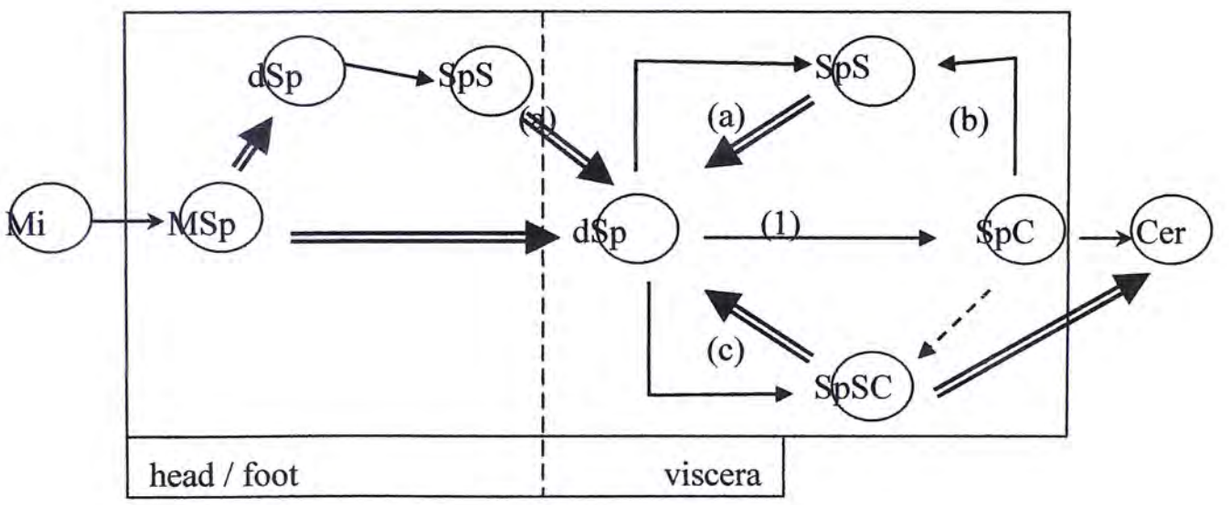


Fig. 2. The intramolluscal larval development of Schistosome. Mi, miracidium; MSp, mother sporocyst; Cer, cercariaed; Sp, daughter sporocysts for several developmental pathways are possible: cerariaogeneous sporocysts (SpC) producing only cercariae (1); sporocystogenous sporocysts (SpS) producing a new generation of daughter sporocysts (dSp) either by direct sporocystogenesis (a); by sporocystogenesis after cercariogenesis (b); or by stimultaneous (c) sporocystgenesis after cercariogenesis (SpSC).

1.2.2.4 Cercaria

The cercariae leave the snail following a daily rhythm. The maximum emission of cercariae from snail of *S. japonicum* of Chinese origin only occurs during the photophase (Theron and Xia, 1986), but that of Phillipines and Formosa origins occur during the scotophase (Kawashiama et al., 1985). However, The mechanism is still unclear.

As miracidium, cercaria is also a free-swimming larva. Cercaria can be simply divided into 2 parts – body and tail. The total length of cercaria is about 0.32 mm. For the part of body, its average length is 140μm and average breadth is 50μm. The swimming cercariae are covered with a single continuous syncytial tegument about 0.5μm thick on the body and 0.2μm thick on the tail. There is a 1 to 2μm dense, labile glycocalyx covered on the syncytial tegument, which may protect the cercariae. Unlike other stages, cercaria does not feed. Glycogen that reserved in body and tail are the energy source of cercaria movement. Glycogen reserved in tail provides the energy for swimming and that reserved in body support the skin penetration (Becker, 1971). For the penetration, 22-35% of glycogen stored in the body of cercaria must be utilized (Lawson and Wilson, 1983). Moreover, cercaria can only survive over the range of 15-35°C. The $t_{1/2}$ of survive at 15°C is about 30 hours, but the $t_{1/2}$ of survive at 35°C is only 8 hours (Lawson and Wilson, 1980).

When a cercaria attaches to the definitive host, it penetrates the host's skin by secreting proteolytic enzymes, such as cercarial elastase (Salter *et al.*, 2002), from its penetration glands, and enters into host's cutaneous capillary vessel. When the cercaria penetrates through, its tail shed off.

1.2.2.5 Schistosomula

The translocation from fresh water to host body requires profound structural, physiological and biochemical adaptations in schistosomula. When a cercaria penetrates into host skin, its tail sheds off (Stirewalt, 1974; McLaren, 1980), and the glycocalyx layer is replaced by a new double-unit membrane on the syncytial surface. (Hockely and McLaren, 1973; Cousin *et al.*, 1981). Moreover, due to the limited glycogen reservoir, schistosomula have to rely on its host for metabolites. After 24 hours of transformation, the energy metabolism of schistosomula is fully anaerobic (Von Kruger *et al.*, 1978; Thompson *et al.*, 1984).

After entering into the host's skin, schistosomula penetrate into capillary venule and travels to lungs by blood flow. Schistosomula leave the lungs via the pulmonary vein and pass via the left side of the heart to be distributed round the host's body. Finally, schistosomula reach the hepatic portal system and stays in the liver (Wheater and Wilson, 1979; Miller and Wilson, 1980; Wilson and Coulson, 1986). Schistosomula develop into adult worms within liver. Mature schistosome losts the migration ability completely and stays at site where it matured for the rest of its life.

1.2.2.6 Adult worms

Schistosoma japonicum is dioecious. The female grows longer compared with male. The male worm is 10-22mm x 0.5-0.55mm in size, and the length of female is about 15-30mm. The oral sucker, ventral sucker and gynaecophoric canal are the typical characteristics of male worm. The male looks like an elongated cylinder, with the gynaecophoric canal deeply grooved out from the ventral sucker posteriad. In the

mature male, spines cover only on the posterior region close to the tail tip, the suckers and the gynaecophoric canal to maintain the position of worm pair against blood flow (Sobhon, *et a.*, 1986). Compared with the external surface of male, that of female worms is simpler and more uniform. After infection, the sex differentiation of schistosomula is recognizable from the sixth day, and develop into sex mature stage within 34 days. During migration inside host body, female and male schistosome meet and mate. The female lives in the gynaecophoric canal of male and develop into sex mature stage together within male. It is very important that female alone cannot develop into reproductive stage without paring with male (Imperia *et al.*, 1980; Torpier *et al.*, 1982). The lifespan of *schistosoma japonicum* in definitive host is 10 – 20 years.

The tegument layer of mature worm is composed by a syncytium having cytoplasmic connections with underlying nucleated bodies. For mature worm, its tegument is rich in lipid, including sphilgomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine.

1.2.3 Genetics of *Schistosoma japonicum*

Schistosoma japonicum is not a good model for the genetic study. Firstly, it is a multi-cellular organism that cannot be cultured in vitro. Secondly, there are six stages in its life cycle, and the differences in morphology and living environment are distinct among them. Study of schistosme is a complex and challengeable task.

In addition, the gain of knowledge on schistosome is important for the

understanding of their biology, mechanisms of drug resistance and antigenic variation that determine the escape from the host's immune attack.

1.2.3.1 Genome analysis

Genetic analysis of the trematode parasite *S. japonicum* has undergone a rapid expansion in recent years (reviewed by Williams and Johnston, 1999; Glora *et al.*, 2000). In order to improve and enhance the collaboration between public and academic institutions, where most of the information of parasite is obtained, and the pharmaceutical industry, the Schsitosoma Genome Project (SGP) was created in 1992.

Recently, the main attentions in schistosome genetic research are focused on (a) development of stage-specific library; (b) identification of interested genes; (c) drawing low-resolution physical map of whole schistosome genome.

Creation of extensive database of expression sequence tags (ESTs) for schistosme promotes the rapid discovery of new genes. In 2000, there were only 1,370 *S. japonicum* ESTs (Glora *et al.*, 2000), but nowadays there are over 45,900 ESTs presented in the database for ESTs (dbEST release at 1 August 2003) [http://www.ncbi.nlm.nih.gov/dbEST/index.html]. ESTs is also useful in database homology searches against either DNA or protein sequences, in an attempt to identify those genes from which they were derived (Adams *et al.*, 1991)

The antigenic sequence tag (AST) is used as a new approach to identify genes directly involved in parasitism and pathogenesis of schistosomiasis (Zouain *et al.*, 1998). This strategy allows the identification genes potentially coding novel antigen

directly. Catepsin L and B (Dalton *et al.*, 1996) and 26kDa GST (Wright *et al.*, 1991) are the typical examples resulted from AST strategy.

In the development of schistosoma antigens, except those are shared among the life cycle stages, stage-specific antigens must be considered separately. Stage-specific antigens elicit separate, independent response that is important to the specific stage and may have no significance in the other stage. In past, RAP-PCR (Melsh *et al.*, 1992) was the most common method used in the identification of differential expression genes. Today, cDNA array are used as a more efficient approach (Hoffmann, 2002). In addition, the combination of techniques such as two-dimensional (2-D) gel electrophoresis, MALDI peptide-mass spectrometry and nanoelectrospray tandem mass spectrometry (Lamond *et al.*, 1997; Pennington *et al.*, 1997) are used to identify proteins from different sexes and stages of schsitosome.

1.2.3.2 Schistosome genome

Schistosome is a diploid eukaryotic organism. For *S. japonicum*, *S. mansoni* and *S. haemotabium*, all of them are have eight pairs of chromosome (reviewed by Short, 1983). There are three pairs of small sized, three pairs of medium chromosome and two pairs of large size chromosomes. Female schistosome is heterogametic, as it contains a ZW pair of chromosomes. For the male, it contains ZZ pair of chromosomes. The estimated genome size is 270Mb and composed of 60% repetitive sequence (Simpson *et al.*, 1982; Simpson *et al.*, 1987).

1.2.4 Tegumental membrane of Schistosomes

Unlike certain protozoa parasites those hide within host cells, schistosomes are exposed to the circulating component and the immune system. The surface of parasite presumably possesses mechanism to evade immune attack. Until now, most of studies of schistosome surface are focused on *S. mansoni.* Presumably, the diagram of constructed for *S. mansoni* can apply into the other species of schistosome, such as *S. japonicum.*

The ultranstructure of schistosome has been studied for many years (reviewed by Kusel and Gordon, 1989; Dorsey *et al.*, 2002). During the lifespan in its definitive host, schistosome is covered by a syncytium called the tegument (Hockely, 1973). The schistosome tegumental membrane is composed by two membranes (Caulfield *et al.*, 1980). The outer membrane facing the host is simply a lipid bilayer consisting of sterol and phospholipid, and the inner one plays a role in ion transport (Fetterer *et al.*, 1980; Podesta, 1983)

In order to protect the parasite form immune attack, one of the approaches is to acquire host antigen from serum or blood cells to mimics the host. The studies for interaction of parasite and eosinophils, neutrophils, monocytes and erythrocytes indicate that all of these cell types can be lysed on the surface of the parasite and fused in the outer membrane (Wislon and Barnes, 1979; Caulfield *et al.*, 1982; Capron and Dessaint, 1985; Kerrow *et al.*, 1985). The identification of lipoprotein receptors on the surface of schistosomula also supports this hypothesis (Rumjanek and McLaren, 1981). The outer membrane of parasite contains mainly phopholipids and cholesterol derived from the host as well as absorbed host proteins and lipids

(Rumjanek and Simpson, 1980). The true parasite molecules, proteins and antigenic glycolipids, are probably only a small fraction of the outer membrane.

The membrane turnover is very important in defense mechanisms utilizing by schistosome to avoid damage caused by host immune attack. The parameter of membrane turnover is difficult to measure *in vivo*, therefore, most of studies of membrane turnover was focused on *in vitro* (Kusel and Mackenzie, 1975; Wislon and Barnes, 1977; Simpson *et al.,* 1981). Moreover, the membrane cannot be considered as a single unit in terms of turnover, as the relative turnover rates of individual membrane proteins are different (Cordeiro *et al.*, 1984).

1.3 Pathology of Schistosomiasis

Schistosomiasis is caused by adult schistosome depositing eggs in blood vessels surrounding the bladder or gut of the infected hosts. Every year, more than two hundred million people are infected and six hundred million people are at the risk of infection (WHO report). Schistosomiasis is not caused by parasite worms themselves. Most of the disease manifestations arisen in host responses to the larval miracidia contained eggs. There are up to 3,500 eggs produced by one pair of *S. japonicum* per day. Most of eggs are trapped within intestinal and hepatic tissues. Those retained eggs induce a cell-mediated granulomatous reaction that accumulates to cause the pathology of chronic diseases.

1.3.1 Acute Schistosomiasis

The acute schistosomiasis appears on an average of 41.5 days after first infection

or a large re-infection (reviewed by Kane and Most, 1948; Chen and Mott, 1989). It relates to the initiation of egg laying by female worms. Patients present with coughing, fever, muscle pain, headache, and a tender enlarged liver.

1.3.2 Intestinal Disease

Accumulated eggs in intestinal tissue form clusters that induce mucosal inflammation, hyperplasia, ulceration, microabscess formation, blood loss and pseudopolyposis (Chen *et al.*, 1978; Cheever and Duvall, 1982; Chen, 1991). In severe cases, intestinal schistosomiasis can develop into colorectal cancer (Abanilla, 1986; Bergquist, 1992).

1.3.3 Hepatosplenic Disease

Schistosme eggs trapped in presinusoidal venules within liver of host body may induce granulomatous inflammation, fibrosis, venous obstruction, portal hypertension and splenomegaly. As a consequence, the liver enlarges (Symmers, 1903; Olds *et al.*, 1996).

1.3.4 Cerebral Schistosomiasis

Cerebral schistosomiasis is caused by the accumulation of parasite eggs in the host nervous system (Warren, 1973). Until now the mechanism of egg deposition in central nervous system is unknown. In China, up to 4.3% schistosomiasis patients have cerebral schistosomiasis (Kane and Most, 1948; Chen, 1991)

1.4 Treatment of Schistosomiasis

1.4.1 Chemotherapy

Until now, the treatment of schistosomiasis can only rely on chemotherapy. Praziquantel is the drug that most commonly used in schistosomiasis treatment (reviewed by Cioli and Pica-Mattoccia, 2003). Praziquantel is tolerated and effective in all the ages of patients and in all the forms of schsitosomiasis. The cure rate of this drug is very high: 85% for *S. hematobium*; 63-85% for *S. mansoni*; 80-90% for *S. japonicum*; 89% for *S. intercalatum* (reviewed by Wegner, 1984). The side effect of praziquanel is very mild and transient, such as headache, usually disappear within 24 hours (Jaoko *et al.*, 1996; Berhe *et al.*, 1999). Although, there is no drug resistance strain reported in *S. japonicum,* the praziquantel resistance strains were reported in *S. mansoni* recently (Ismail *et al.*1996 and 1999).

Artemether, another effective drug, is a derivative of the anti-malarial drug artemisinin (reviewed by Xia *et al.*, 2002). The drug is active in against *S. japonicum*, *S. mansoni*, and *S. haematobium* with low toxicity (Utzinger *et al.*, 2000; Xiao *et al.*, 2000; Yang *et al.*, 2001). Combined with praziquantel in schistosomiasis treatment, its overall effect can be enhanced (Xiao *et al.*, 2000).

1.4.2 Schistosoma Vaccine

Nowadays, the control of schistosomiasis is still relied on snail (intermediate host) eradication, and chemotherapy. However, the costs of drugs, diagnosis and the delivery of treatment are high, especially in developing countries. In addition, the

re-infection occurs rapidly and treatment must be repeated at frequent intervals with the potential to lead to drug resistance. The praziquantel resistance strains have been reported in *S. mansoni.* Schistosome is a kind of non-replicating organism in the definitive host. Therefore, a vaccine-induced immune response could strongly decease human pathology and transmission levels.

It is well known that the infection of attenuated cercariae in animal model can evoke protective immunity against a challenge infection (reviewed by McManus, 1999). The experiment has been successfully demonstrated in rodents, pigs, and water buffaloes by either *S. japonicum* or *S. manosoni* (Bickle *et al.*, 1985; Moloney *et al.*, 1985). However, the protection against schistosome parasites appears to be species-specific (Bickle *et al.*, 1985; Moloney *et al.*, 1985). In addition, for Chinese and Philippine strains of *S. japonicum*, the protection is even strain-specific (Moloney, Garcia and Webbe, 1985; Zhang *et al.*, 1999). In China, the attenuated *S. japonicum* cercarial vaccines have been tested in animal models in field condition (Shi *et al.*, 1990 and 1993). Because of the difficulty and impracticality of producing quality controlled, reproducible batches of vaccines on a large scale and the associated safety consideration, the attenuated cercaeria vaccine is not suitable for using in human or for widespread veterinary application (McManus, 1999).

As a consequence, people are focused on the identification of parasite antigens that may be involved in inducing protective immune responses. Until now, there are over 100 antigens developed, but none can reach the level close to sterile immunity seen after vaccination with attenuated cercariae (reviewd by Bergquist, 1998; Capron *et al.*, 2002; Bergquist *et al.*, 2002). Certain antigens that can provide significant pathology reduction (e.g. worm intensity reduction) are used as an adjunct for

chemotherapy (El Ridi *et al.*, 2001).

1.5 GPI anchor

A group of proteins that attached on the outer membrane of eukaryotic cell via glcosylphosphatidylinositols (GPI) anchor is called GPI anchored proteins. Based on their structures, the GPI anchored molecules can be divided into two major groups. One is the cell surface glycoprteins bearing GPI anchors and the other one is protein free – glycosylated phosphatidylinositols PI(s).

1.5.1 Function of GPI anchored proteins

In the last decade, various functions of GPI anchored proteins have been identified, particularly in mammalian and protozoan (reviewed by Cross, 1990; Ferguson, 1992; Malcolm *et al.,* 1993).

In both mammalian and protozoan, the most fundamental function of GPI anchors is to provide a stable linkage between anchored proteins and membranes. Compared with hydrophobic polypeptide domains, the GPI anchor is a more stable anchor. Most GPI anchored proteins exhibit low turnover rates, for example Thy-1 (Bulow, *et.al.*, 1989; Lemansky *et al.*, 1990; Seyfang *et al.*, 1990). It is believed that GPI anchored proteins are endocytosed (Webster and Grab, 1988; Webster *et al.*, 1990) and recycled (Seyfang *et al.*, 1990) via clathrin-dependent endocytic pathway (Lemansky *et al.*, 1990). In addition, the presence of GPI anchor provides a selective release of anchored protein via the action of glycosylphosphatidylinositol-specific phospholipase. Until now, there are two kinds of

glycosylphosphatidylinositol-specific phospholipases acted on GPI anchors have been identified: glycosylphosphatidylinositol-specific phospholipase C (GPI-PLC) (Redpath *et al.*, 1998) and glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) (LeBoeuf *et al.*, 1998; Xiaotong *et al.*, 2002). The releasing of variant surface glycoproteins (VSG) in *T. brucei* is the typical example of GPI-PLC (Mensa-Wilmot *et al.*, 1993).

In mammalian cells, the GPI anchored proteins play a role in the formation of microdomains (Lisanti *et al.*, 1990; Brown and Rose, 1992; Rodriguez-Boulan and Powell, 1992; Hannan *et al.*, 1993). One of the examples of functional GPI microdomains is caveolae. Caveolae, which is formed by GPI anchored proteins, is responsible for potocytosis (reviewed by Anderson *et al.*, 1992; Hooper, 1998; Mineo and Anderson, 2001). Potocytosis is a psuedo-endocytic pathway that is important in small and large molecules transportation.

For parasitic protozoa, GPI anchors play important roles in the adaptation inside host body. For example, GPI anchor allows the intensive packing of variant surface glycoprotein (VSG) without using up the membrane space in African trypanosome (reviewed by Pays *et al.*, 2001). In *T. brucei*, the protective glycocalyx layer, which is the macromolecule diffusion barrier, is formed by the modification of oligosaccharide side chains on GPI anchors (Metcalf *et al.*, 1987; Homans *et al.*, 1989; Tomlison *et al.*, 1992; Ferguson *et al.*, 1993).

In addition, the synthesis pathway of GPI anchor may play a role as second messengers in the signaling cascades triggered by specific hormones and growth factors (reviewed by Field, 1997; Brodbeck, 1998).

1.5.2 Synthesis of GPI anchor

Based on the structures of different GPI anchors from different species, the main core structure of GPI anchor is conserved throughout evolution (Ferguson et al., 1988; McConville and Ferguson, 1993). The core structure of GPI anchor is: the C-terminal of protein links via ethanolamine phosphate (EtN-*P*) to a tetrasaccharide of Manα1-2Manα1-6Manα1-4GlcN, that binds to myo-inositol in α1-6 linkage (reviewed by Ikezawa, 2002). However, for different species, the GPI lipid moieties are different. The myo-inositol ring is linked to sn-1,2-diacylglycerol or sn-1-alkyl-2-acylglycerol to form a common glycosylated phosphatidylinositol (PI) in most cases. In protozoa, sn-1,2-diacylglycerol or sn-1-lyso-sn-2-acylglycerol is found. In Yeast, the lipid is usually sn-1,2-diacylglycerol or a ceramide (Roberts *et al.*, 1988; Field *et al.*, 1991; Treumann *et al.*, 1995). The (Fig. 3) shows the core structure of GPI anchors.

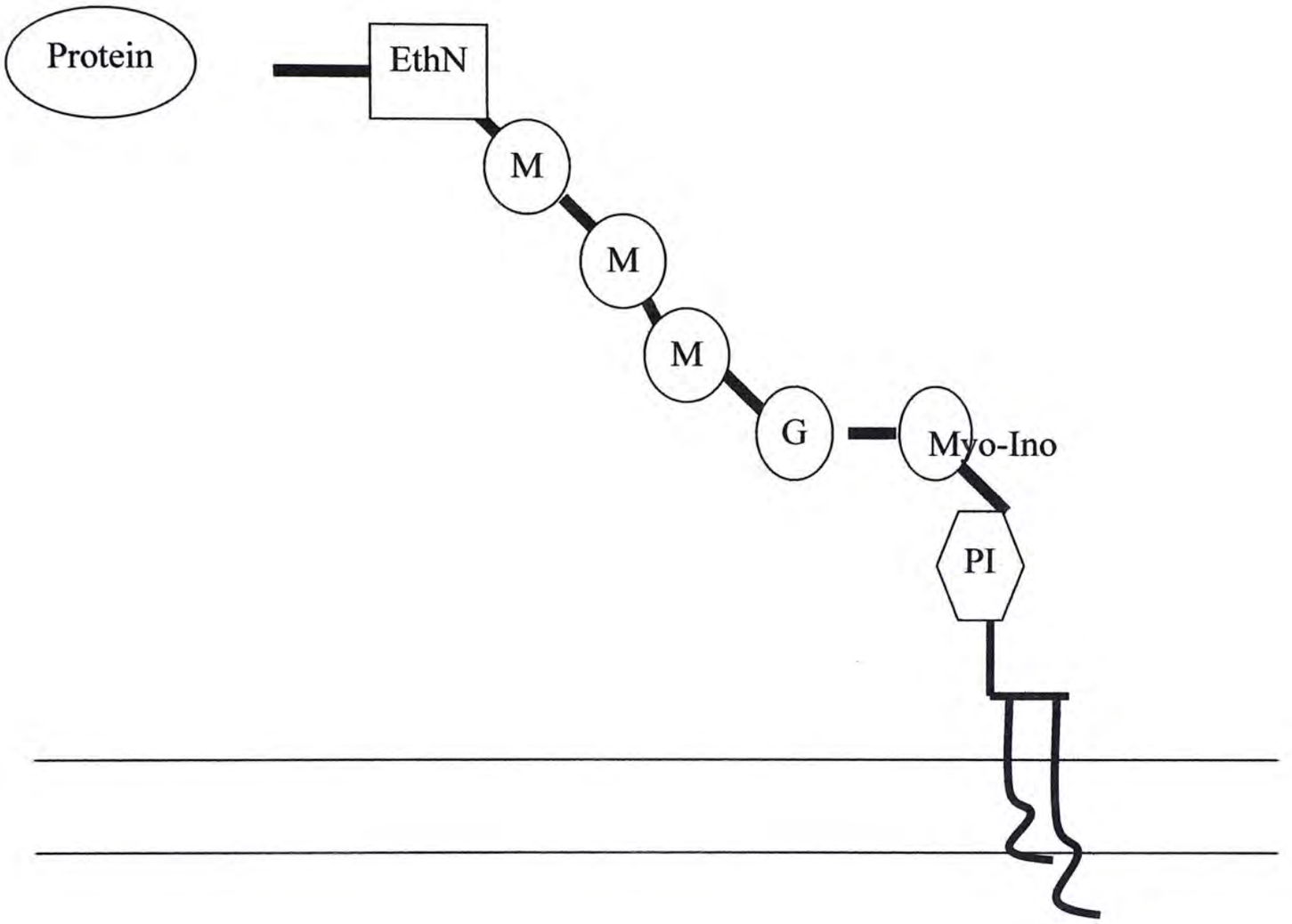


Fig. 3. GPI anchored protein on Eukaryotic cells (Ikezawa, 2002). GPI membrane anchors, which are found on all eukaryotic cells, contain a conserved core structure of EtN-*P*-6Manα1-2Manα1-6Manα1-4GlcNα1-6PI. EtN: phosphoethanolamine; Man: mannose; G: glucosamine; *myo*-Ino: *myo*-inositol, hydrophobic anchor region: monoacylglycerol, diradylglycerol (diacyl or 1-alkyl-2-acylglycerol), and ceramide.

In the synthesis of GPI anchor (Fig. 4), the first step is the formation of GlcNAc-PI. GlcNAc is transferred from UDP to PI to form GlcNAc-PI under the action of α1-6 GicNAc transferase. In mammalian cells, three homologous are identified that involved in the step: phosphatidylinositol glycan class A (PIG-A) (Miyata *et al.*, 1993), PIG-C (Inoue *et al.*, 1996), and PIG-H (Kamitani *et al.*, 1993). In the next step, the GlcNAc-PI is de-N-acetylated into GlcN-PI by de-N-acetylase, PIG-L (Nakamura et al., 1997). Moreover, enzyme is only activated by GTP in mammalian (Steven, 1993). Due to the PIG-L locating on the cytoplasmic side of ER membrane, this GPI synthesis step does not process within ER (Watanabe *et al.*, 1999). The third step in GPI synthesis is the acylation of the two position of the inositol ring of GlcN-PI (Costello and Orlean, 1992). As a result, Man1-GlcN-(acylinositol)PI is formed. It is believed that the reaction is under the action of acyl coenzyme A (acyl-CoA) in the presence of palmitoyl-CoA. Recently, the gene – GWT1, encoding the enzyme, has been cloned in yeast (Umemura *et al.*, 2003). After acylation, three mannose residues are added to the GPI precursor to form Manα1-2Manα1-6Manα1-4GlcNα1-6PI. Each mannose is transferred to GPI precursor from the common sugar donor, dolicholphosphate mannose (Dol-P-Man), by its specific GPI mannosyltransferase (GPI-MT-I, II and III) (Menon *et al.*, 1990). In mammalian, the GPI-MT-I and GPI-MT-III genes have been identified and named as PIG-M (Maeda *et al.*, 2001) and PIG-B (Takahashi *et al.*, 1996) respectively. Although there are several putative genes coding for GPI-MT-II that have been discovered (Amado *et al.*, 1999), the functions in GPI anchor synthesis are not proved, such as Not56D (Korner *et al.*, 1999). The final step of GPI anchor synthesis is the addition of ethanolamine phosphate (EtN-P). The addition of EtN-P to the terminal mannose is essential step and provides the bridge between GPI anchor and anchored protein. EtN-P is transferred to the position six of the terminal mannose under the

function of phosphatidylethanolamine transferase: PIG-F and PIG-O (Hong *et al.*, 2000). Except the main structure, the GPI anchor contains EtN-p side chains that are variable in different species. For both mammalian and yeast cells, there is an EtN-P side chain on the first mannose of GPI anchor. The first EtN-P is added to the position two of first mannose residue under the action of PIG-N (Hong *et al.*, 1999). This addition of EtN-P occurs at the stage of Man1-GlcN-(acylinositol)PI (Homans *et al.*, 1988; Puoti *et al.*, 1991; Puoti and Conzelmann, 1993; Canivenc-Gansel *et al.*, 1998). Another EtN-*P* side chain is attached at the position six of the second mannose (Deeg *et al.*, 1992; Kamitani *et al.*, 1992). But until now, the donor of EtN-P is still unknown in neither mammalian cells nor protozoa cells. After the completion of GPI anchor main core, the fatty acid moiety of GPI anchor is modified by arylacetamide deacetylase (Yamazaki *et al.*, 1993). In *T. brucei*, two acyl groups in the diacylglycerol moiety of PI are replaced with myristoyl groups by sequential deacylation-reacylation (Masterson *et al.*, 1990; Doering *et al.*, 1994).

For the proteins that can attach to GPI anchor, must contain three features: the first one is an amino-terminal hydrophobic signal peptide, which will be removed after the protein enters into ER lumen (reviewed by Udenfriend and Kodukula, 1995); the second one is a carboxyl-terminal that can locate the protein on ER membrane and can be removed during anchor attachment process; the third requirement is that all GPI anchored protein must carry a triplet of amino acids (Kodukula *et al.*, 1993) at the GPI cleavage/attachment site – ω site. In mammalian system, there are four genes responsible for the protein attachment process have been identified: GPAA1 (Inoue *et al.*, 1999), PIG-K, PIG-S, PIG-T (Vainauskas *et al.*, 2002).

It is already known that the initial step of GPI synthesis starts on the cytoplasmic

face of the rough ER membrane, and the final step takes place on the lumen side. However, the exact step of GPI precursor, which turns into ER lumen, is unknown.

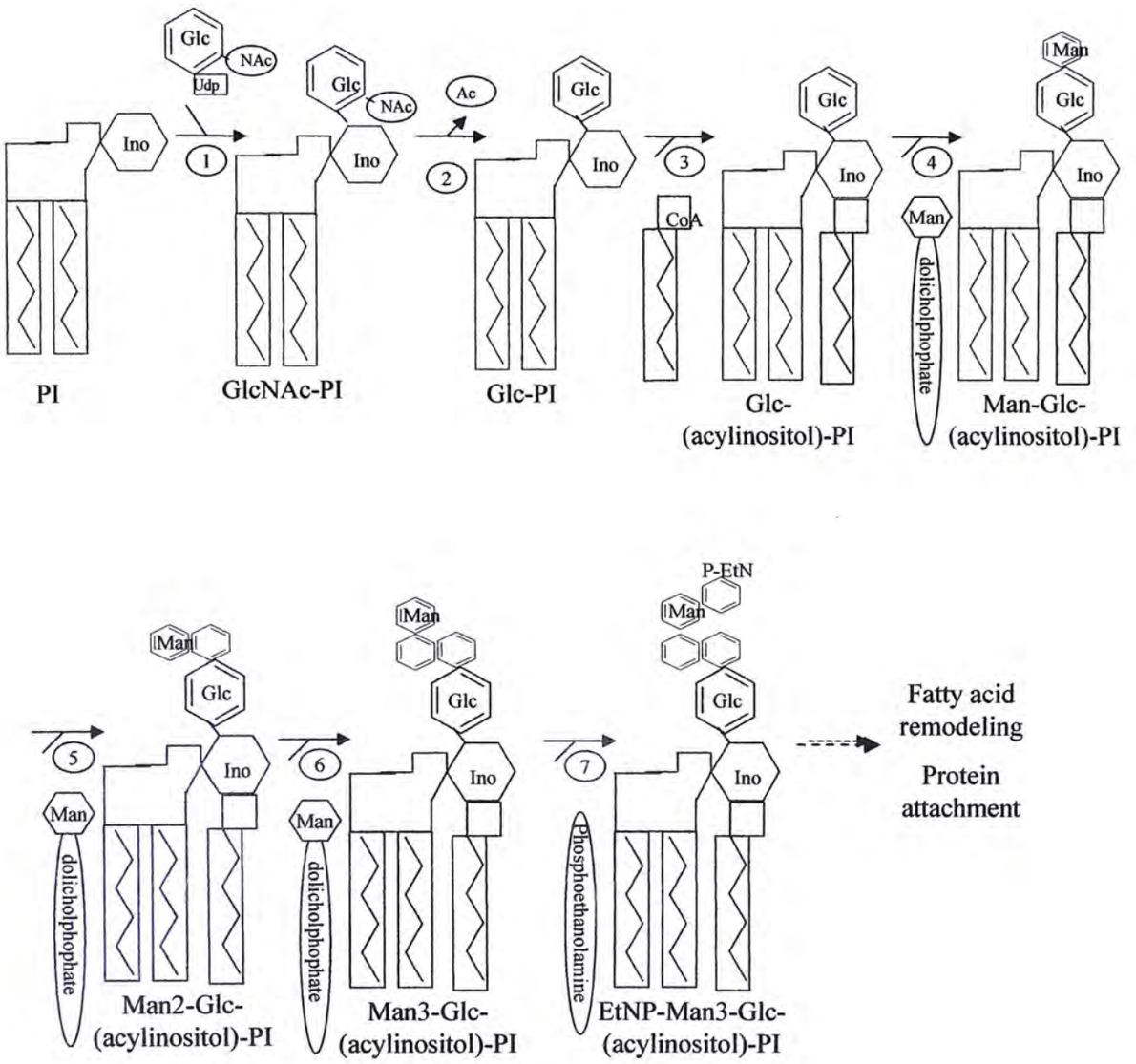


Fig. 4. The GPI Biosynthetic Pathway. The graph presents the steps of the GPI anchor synthesis pathway in eukaryote. In brief, GlcNAc is transferred to PI from GlcNAc-PI, (1). After deacetylation, it transforms into GlcN-PI, (2). It is then acylated into GlcN-(acylinositol)-PI, (3). After that, three mannose residues are added from dolicholphophate mannose to the precursor subsequently, (4), (5), (6). At last, ethanlolamine is transferred to the terminal mannose to form EtN-P-Man_3-GlcN-(acylinositol)PI, (7). After fatty acid remodeling, the GPI anchor is ready for the protein attachment. (Tiede *et al.*, 1999)

1.5.3 Phosphatidylinositol Glycan, Class N

During the synthesis of GPI anchor, a EtN-P is attached to the position two of first mannose on the Man1-GlcN-(acylinositol)PI stage under the action of phosphatidylethanolamine transferase. Recently, genes encoding the enzyme have been identified and functionally proved in mammalian and yeast cells. In mammalian cells, the gene is named PIG-N (Hong *et al.*, 1999); in yeast, the gene is named MCD4p (Gaynor *et al.*, 1999). Moreover, putative genes that are homologous to PIG-N are also found in *Caenorhabditis elegans* and *Arabidopsis thaliana* (tblastn search). In both PIG-N and MCD4 proteins, their N-terminals are highly hydrophobic and their C-terminal contains "KKXX" ER retrieval motif (Jackson *et al.*, 1993; Gaynor *et al.*, 1994). The C-terminal of PIG-N ends with "KKLQ", and the C-terminal of MCD4p carries "KKTQ". The localization of MCD4p in ER has been demonstrated by co-immunoprecpitation (Gaynor *et al.*, 1999). In addition, the alignment result of those phosphatidylethanolamine transferases indicates these enzymes contain conserved domains of mammalian phosphodiesterases and nucleotide pryophosphatases.

1.6 The role of GPI anchor proteins in Schistosome

GPI anchored proteins play an essential role in the immune evasion of protozoa parasite. The best-characterized example is the VSG of trypanosomes. Trypanosome is covered with a layer of GPI anchored glycoproteins, which can be shed off under the action of PI-PLC. These glycoproteins are also called variant surface glycoproteins (VSG) that contain variable antigen types (VAT). The trypanosome evades the host's immune response by a process termed Antigenic Variation in which

the complete surface coat is sequentially replaced by a similar, but antigenically distinct, surface coat (reviewed by Donelson, 2003). A similar antigenic variation phenomenon does not observed in schistosome. Although several major antigens of *S. mansoni* are GPI anchored proteins, such as 38 kDa, 32 kDa, and 18 kDa proteins (Pearce and Sher, 1989). On the other hand, PI-PLC selectively released the 200-kDa antigen from the surface of adult schistosomes (Sauma *et al.*, 1991) suggests that the trematode lipases may be involved in immune evasion via antibody-stimulated antigen release (Hawn and Strand, 1992) and this strategy of immune evasion may be disrupted by praziquantel (Brindley *et al.*, 1989; Sauma *et al.*, 1991).

Schistosome develops its unique mechanism to against the attack from host immune system. It is believed that the interaction between host molecules and schistosome plays an important role in the survival of the parasites. The tegument layer of schistosome can bind a wide range of host molecules including gycolipid moiety and proteins, such as the histocompatiblity antigens and cholesterol (Goldring *et al.*, 1976; Sher *et al.*, 1978; Ramalho-Pinto *et al.*, 1978; Smith and Kusel, 1979; Doenhoff *et al.*, 2002). These host lipoproteins cover on the parasite surface to mimic host cells. Therefore, the parasites can escape from host immune attack. Although the mechanism of host protein acquirement is still unclear, many evidences indicate that the GPI anchored LDL binding proteins (Xu and Caulfield, 1992) are involved in the acquirement of these low-density lipoproteins (LDL) or very low-density lipoproteins (VLDL). The GPI anchored LDL binding proteins are different for *S. mansoni* and *S. japonicum*. In adult worm of *S. mansoni*, 60, 35, 14 kDa and a 45 kDa doublet LDL binding proteins have been reported (Samuelson *et al.*, 1982; Rumjanek *et al.*, 1985; Tempone *et al.*, 1997), but there are 17.8 and 15.7 kDa LDL binding proteins reported in schistosomula (Xu and Caulfield, 1992). A 43 kDa LDL binding protein is reported

in adult worm of *S. japoncium* (Rogers *et al.*, 1990). As the schistosome cannot synthesize cholesterol or fatty acids, the acquired host lipoproteins are not only play a role in immune evasion, but also the source of cholesterol. These major cholesterol-carrying lipoproteins in human plasma are "captured" by LDL binding proteins and ingested by the parasite (Chiang and Caulfield, 1989; Chiang and Caulfield (b), 1989; Bennett *et al.*, 1991).

The tegument layer is the main rout for the absorption of glucose, amino acids and other nutrients of small size (Cornford and Huot, 1981; Cornford *et al.*, 1983; Bryant, 1993; Camacho and Agnew, 1995). As there is no reported endocytosis in schistosome, potocytosis may play a role in the molecule transportation. Caveolae structure, a subset of GPI anchored surface proteins, is responsible for the potocytosis and physically associated with detergent-insoluble gycosphingolipid-enriched membrane domains (DIGs) (Hope and Pike, 1996). In *S. mansoni*, the caveolae-like structure is found (Racoosin *et al.*, 1999). The detergent-insoluble complexes (DIC) are isolated from schistosome tegument layer with sucrose density gradient centrifuge. In these DIC, GPI anchored proteins SmAP (Payares, *et al.*, 1984), Sm200, Sm23 (Lee, 1995) and a protein recognized by anti-human caveolae antibody are identified. Moreover, the caveolae-like structure is also observed under transmission electron microscope.

Acetylcholinesterase (AChE) is a GPI anchored protein that presents in muscle and the tegument of schistosome (Gear and Fripp, 1974; Camacho *et al.*, 1996). Enzymes located in muscle are probably involved in regulation of access of the neurotransmitter acetycholine (ACh) to acetycholine receptors (AchR) at cholinergic synapses (Legay, 2000). The role of surface AChE in schistosome is not very clear, and need further analysis. In nematode parasite *Nippostrongylus brasiliensis*, AChE

can be secreted into the host lumen to interfere with expulsion of the parasite from the host (Hussein *et al.*, 1999). But in *S. mansoni* and *S. heamatobium*, AChE may play a role in "non-classical" cholinergic system to regulate glucose uptake from host on tegument layer (Pax *et al.*, 1984; Camacho *et al.*, 1995; Camacho *et al.*, 1996; Jones *et al.*, 2002). In the "non-classical" system, AChE probably limits the interaction of ACh and its receptor - AChR that regulate host glucose uptaking process (Camacho and Agnew, 1995). In addition, those ACh are derived from host bloodstream (Kawashima *et al.*, 1993; Fujii *et al.*, 1995).

1.7 Aim of study

S. japonicum is a blood stream parasite that cause schistosomiasis which is the second most prevalent parasitic disease in the world. It is epidemic in 74 countries and territories with over 200 millions people affected. Until now the treatment of schistosomiasis is still relied on chemotherapy, and no vaccine can effectively against the parasitic infection. Recently, the reported drug resistance strains in S. mansoni indicate that development of new treatment approach and drug discovery to against schistosomiasis is an urgent task for scientists.

In this study of the *S. japonicum* cercaria cDNA library. I have identified a PIG-N homologous gene. |The PIG-N gene is involved in the GPI anchor synthesis. Although the GPI anchored proteins play important role in parasite survive and evasion mechanism from host immune response, GPI anchor synthesis gene has not been identified on the parasites. The aim of the project is to study the molecular structure of the *S. japonicum* PIG-N gene and to demonstrate the biochemical function of its gene product. Studying of *S. japonicum* PIG-N homologous gene will

help us to understand more in the GPI anchor synthesis pathway in schistosome. In addition, it may provide a new direction for anti-schistosomiasis drug screening.

Chapter Two Materials and Methods

2.1 MATERIALS

2.1.1 Cell lines and Bacterial Strains

1. 3T12 murine cell line

The 3T12 cell line was a gift from Dr. K.N. Leung at the department of Biochemistry, the Chinese University of Hong Kong. The 3T12 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS, Gibco), and 50 U/ml penicillin, 50μg/ml streptomycin, and 10 μg/ml neomycin. The cultures were maintained under a humidified atmosphere of 95% air / 5% CO_2 at 37°C.

2. F9 murine embryonal carcinoma cell line

The F9 cell line was a gift from Dr Taroh Kinoshita at the Department of Immunoregulation, Resarch Institute for Microbial Diseases, Osaka University, Japan. The F9 cells were cultured in high glucose Dulbecco's Modified Eagle Medium supplemented with, 10% 10% fetal calf serum (FCS, Gibco), and 50 U/ml penicillin, 50μg/ml streptomycin, and 10μg/ml neomycin. The cultures were maintained under a humidified atmosphere of 95% air / 5% CO_2 at 37°C.

3. F9 PIG-N double knock cell line

The F9 PIG-N double knock out cell line was a gift Dr Taroh Kinoshita at the Department of Immunoregulation, Resarch Institute for Microbial Diseases, Osaka University, Japan. In the F9 PIG-N double knock out cell line, the mouse PIG-N gene

was disrupted.

4. *Escherichia coli*, DH5α

5. *Escherichia coli*, BM25.8

6. *Escherichia coli*, XL-1 Blue

2.1.2 Chemicals

1. Agar (Bacteriological grade)	Ajax 863
2. Agarose, Seakem LE	FMC50004
3. Ampicillin	Sigma A9518
4. Calcium chloride, hydrated form	Sigma C3881
5. Cesium chloride	Sigma C4036
6. Chloroform	Ajax 152
7. Chloramphenical	Sigma C0378
8. Diethyl pyrocarbonate, (DEPC)	Sigma D5758
9. Ethidium bromide	Sigma E8751
10. Ethylenediamine-tetraacetic acid (EDTA)	Sigma ED2SS
11. Ethanol, absolute	Ajax 214
12. Gelatin	SigmaG9382
13. Geneticin (G418)	GibcoBRL 11811-031
14. Glycerol	Sigma G5516
15. Glycine	Sigma G7403
16. Guanidine thiocyanate	Fluka 50990

17. Hydrochloric acid, 32%	Ajax 265
18. Hygromycin B	Clontech 8057-1
19. Isopropyl-β-D-thiogalactopyranoside (IPTG)	Sigma I6758
20. Kanamycin	Sigma K0879
21. Magnesium chloride	Sigma M9272
22. Magnesium sulfate	Sigma M2773
23. Maltose	Sigma M2250
24. Manganese chloride, tetrahydrate	Sigma M3634
25. β-mercaptoethanol	Sigma M7154
26. Mgnesium sulphate ($MgSO_4$)	Ajax 303
27. Paraformaldehye	Sigma P6148
28. Sodium acetate	Sigma S8750
29. Sodium azide	Sigma S2002
30. Sodium bicarbonate	Sigma S8875
31. Sodium Chloride	Sigma S9625
32. Sodium phosphate	Sigma S0876
33. Tris-Acetate-EDTA (TAE) buffer	Sigma T9650
34. Tetracycline	Sigma T3258
35. Thimersol	Sigma T8784
36. Trizma Base	Sigma T6791
37. Tryptone	Oxoid L42
38. Tween 20	Sigma P1379
39. Urea	Sigma U5128
40. 5-Bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal)	Boehringer Mannheim 1585002
41. Yeast extract	Oxoid L21

2.1.3 Kits and Reagents

1.	Advantage® 2 PCR Kit	Clontech K1910-Y
2.	dRhodamine terminator cycle sequencing FS ready RX kit	Perkin Elmer 403044
3.	Qiaex II gel extraction kit	Qiagen 20021
4.	SMART$_{TM}$ cDNA Library Construction kit	Clontech k1051-1
5.	SMART$_{TM}$ RACE Amplification Kit	Clontech K1811-1
6.	Wizard® *Plus* SV Minipreps DNA Purification System	Promega A7500
7.	DMRIE-C reagent	GibcoBRL 10459-014
8.	HiDi formamide	Perkin Elmer 4311320

2.1.4 Nucleic acids

1.	ATP	Pharmacia 27-1006-01
2.	dNTPs	Pharmacia 27-2035-03
3.	pBluescriptII KS (+)	Strategene
4.	pTripleEX 2	Clontech
5.	pTRE2	Clontech
6.	pEGFP-N1	Clontech
7.	pTK-Hyg	Clontech

2.1.5 Reagents for Cell culture

1.	Fetal bovine serum	GibcoBRL 16000-044
2.	Opti-MEM I reduced serum medium	GibcoBRL 31985-062
3.	RPMI 1640	GibcoBRL 23400-021

4. Dulbecco's phosphate-buffered saline — GibcoBRL 21600-010
5. Dulbecco's modified eagle medium — GibcoBRL 12800-017

2.1.6 Solutions

1. Agarose gel, 1%	1% (w/v) agarose in 1x TAE buffer.
2. FACS fixative buffer	1% paraformaldehyde in 0.85% NaCl.
3. FACS medium	2% fetal bovine serum and 0.05% sodium azide in 1x Dulbecco's phosphate-buffered saline solution
4. LB broth / agar	10g NaCl, 10g Tryptone, 5g Yeast Extract and 15g bacterograde agar (for LB agar only). Add with 1 litre of distilled water and autoclaved.
5. LB+Amp100 agar / broth	LB agar/broth was prepared and added with filter-sterilized ampilicin to final concentration of 100μg/ml.
6. LB+Kan agar / broth	LB agar/broth was prepared and added with filter-sterilized kanamycin to final concentration of 50μg/ml.
7. LB+Kan+Cam agar / broth	LB agar/broth was prepared and added with filter-sterilized kanamycin to final concentration of 50μg/ml and filter-sterilized choramphincol to final concentraion of 50μg/ml.
8. LB+$MgSO_4$ agar / broth	LB agar/broth was prepared and added with filter-sterilized $MgSO_4$ to final concentration of 10mM.
9. LB+$MgSO_4$ +Maltoseagar / broth	LB agar/broth was prepared and added with filter-sterilized $MgSO_4$ to final concentration of 1mM and filter-sterilized Maltose to final concentration of 0.2%.

10. LB+$MgSO_4$ soft agar	10g NaCl, 10g Tryptone, 5g Yeast Extract and 7.2g bacterograde agar. Add with 1 litre of distilled water and autoclaved. Add with filter-sterilized $MgSO_4$ to final concentration of 10mM.
11. LB+Tet agar / broth	LB agar/broth was prepared and added with filter-sterilized tetracycline to final concentration of 15μg/ml.
12. RF 1 solution	10mM RbCl, 50mM $MnCl_2$, 30mM potassium acetate (pH 7.5), 10mM $CaCl_2$, 15% (w/v) Glycerol. Adjust pH to 5.8 with 0.2M acetic acid and sterilize by filtration through a pre-rinsed 0.22μm membrane.
13. RF 2 solution	10mM MOPS, 10mM RbCl, 75mM $CaCl_2$, 15% (w/v) Glycerol. Adjust pH to final pH 6.8 with NaOH and sterilize by filtration through a pre-rinse 0.22μm membrane.
14. SOB solution	2% Bacto-tryptone, 0.5% Bacto yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$.
15. SOC solution	SOB medium plus 20mM glucose solution
16. 1M MgSO4	Dissolve 24.65 g of MgSO4•7H2O in 100 ml of deionized H_2O. Filter sterilize.
17. 1x lambda dilution buffer	100 ml 10X Lambda dilution buffer and 5 ml 2% Gelatin (0.01% final concentration). Add H2O to a final volume of 1 L. Autoclave and store at 4°C.
18. 10x lambda dilution buffer	58.3 g of NaCl, 24.65g of MgSO4•7H2O and 350.0 ml of 1 M Tris-HCI (pH 7.5). Add H2O to a final volume of 1 L. Autoclave and store at 4°C.

19. 20% maltose — Dissolve 20 g of maltose in 80 ml of deionized H2O; bring volume up to 100 ml. Filter sterilize and store at 4°C

2.1.7 Enzymes

1. T4 DNA ligase — Pharmacia 27-0870-04
2. M-MLV reverse transcriptase — Gibco BRL 28025-013
3. rRNasin ribonuclease inhibitor — Promega N2511
4. Thermoprime^{Plus} DNA polymerase; — Biotechnologies #AB-0301
5. AflII — New England Biolab 520S
6. AflIII — New England Biolab 541S
7. BamHI — Pharmacia E0204Y
8. BglII — Pharmacia 27-0946-01
9. BstEII — Promega R06641
10. EcoRV — Promega 144872
11. HindIII — Pharmacia 27-0934
12. SmaI — Promega R221
13. SspI — New England Biolab 132S
14. XbaI — Promega R618G

2.1.8 Primer List

Name of primer	Sequence (5' to 3')
5' CDS primer	$(T)_{25}N_{-1}N$

	(N = A, C, G, or T; N-1 = A, G, or C)
3' CDS primer	AAG CAG TGG TAT CAA CGC AGA GTA C$(T)_{30}N_{-1}N$
SMART II A Oligonucleotide	AAG CAG TGG TAT CAA CGC AGA GTA CGC GGG
10X Universal Primer A Mix (UPM)	Long (0.4 mM): CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT Short (2 mM): CTA ATA CGA CTC ACT ATA GGG C
Nested Universal Primer A	AAG CAG TGG TAT CAA CGC AGA GT
MF520	AAT TAA CCC TCA CTA AAG GG
MF521	TAA TAC GAC TCA CTA TAG GGC GA
MF656	TCC GAG ATC TGG ACG AGC
MF657	TAA TAC GAC TCA CTA TAG GG
MF802	GGA CGA ATC CTG TGG AGT TTG ATA CCG
MF803	CCG ATG GGT CAC CGA TCA GTT TAC G
MF804	GGG ATT GGG ATG CCT AAA AGA CCC G
MF805	CGG ACC GAC AAT ACC AGA ACC CCA A
MF806	TCA AAG CAA CTC CTT ACC CAG
MF807	GGG AAG TCA TGG ATC TGG TT
MF808	GGA CCG ACA ATA CCA GAA CC
MF809	ACC CAT CGG TCG ATT TCT GT
MF834	AGC GGG AAT TCA TCC TGG AGG TC

MF835	GGA TTC AGT TTA TTG GTT CTT TC
MF836	GTA CTT GAC AAA GGA TGA GTA G
MF838	TAT ACA AGT GTA AAG GTA CAA AGT AGC
MF867	ACT TAC ACA TGG CGT AAA CGT TA
MF868	CCT TGA TTC GGT AGG GAC TCT
MF869	GCC CAA TTA AAG ATT AAG TAC AC
MF870	TAT CCG CAT AGC TTT GAT AAT TT
MF871	TGT TTA CTA CTT TCT TCG TGG C
MF872	GCC ACG AAG AAA GTA GTA AAC A
MF873	TTC ATA TAT ACA GTT GGT CAG TG
MF874	CTA CAG CGA ATA CAG CAA AGA AT
MF875	TAT ACT AGC GAT ATT TCC GGT AC
MF876	AAT CCT GCT TTA ATG GCG ATT CT
MF877	GTA GTT GGT TAG ATA TAG GTA CT

2.1.9 Antibodies

1. Anti mouse CD90 (Thy1) G7. BD Biosciences 553016
2. FITC conjugated polyclonal anti-rat IgG BD Biosciences 554016
3. *S. japonicum* infected rabbit serum *

* Provided by Dr. ZhongDao, Wu at Department of Parasitology, Sun Yat – Sen University of Medical Sciences, China.

2.2 Methods

2.2.1 Screening of the *S. japonicum* cercaria stage cDNA library

2.2.1.1 λ phage plating

Fresh XL-Blue bacterial colonies were obtained from glycerol stocks. Certain frozen bacteria-glycerol was scrapped by sterile inoculation loop and streaked on LB/Tet agar plates. The plates were incubated at 37°C overnight to allow development of bacterial colonies. Single colony was then picked up and inoculated into 15ml LB/$MgSO_4$/maltose medium in 150ml flask. It was shaken at 140 rpm at 37°C for overnight until the OD_{600} reached 2.0. The cells were centrifuged for 5 minutes at 5000×g, and then poured off the supernatant was discarded. The pellet was resuspended in 7.5ml of 10mM $MgSO_4$. 500µl of overnight XL1-Blue culture were transferred into a 12 ml Falcon tube containing 1µl (1:1000) diluted λ phage (6.5 x 10^9 pfu/ml). The Falcon tube was incubated in a 37°C water bath for 15 minutes. 2 ml melted LB/$MgSO_4$ soft-top agar was added into the tube. After quickly mixing, the bacteria, phage and soft-top agar mixture were poured onto an LB/$MgSO_4$ agar plate. The LB/$MgSO_4$ agar plate was quickly swirled while pouring to promote even agar distribution. To allow the top to be hardened, the plate was cooled at room temperature for 10 minutes. After that, the plate was inverted and incubated at 37°C for 6 – 18 hours, until the plaques developed.

2.2.1.2 Single plaque isolation

While the plaques were clearly developed, a well-isolated plaque were picked up

by a sterile p-200 pepite tip and transferred into a 1.5ml Eppendorf tube containing 500µl of 1x lambda dilution buffer. And the tube was put at 4°C overnight. Then the tube was shaken at 250 rpm for 1 hour at room temperature. 10µl of chloroform was added into the tube and vortex for 2 minutes. The lysate were centrifuged at 5,000×g for 10 minutes. The supernatant was transferred to a new eppendorf and stored at 4°C.

2.2.1.3 Conversion of Lambda TriplEx to pTriplEx

Fresh BM25.8 bacterial colonies was obtained from glycerol stocks. The frozen bacteria-glycerol was scrapped by sterile inoculation loop and streaked on LB/kan/cam agar plates. The plates were incubated at 37°C overnight to allow development of bacterial colonies. Single colony was then picked up and inoculated into 10ml LB/$MgSO_4$ medium in 150ml flask. It was shaken at 150 rpm at 31°C for overnight until the OD_{600} reached 1.1 – 1.4. 100µl of 1 M $MgCl_2$ was added to the 10ml overnight bacterial culture to reach the 10mM final concentration of $MgCl_2$ and stored at 4°C. 200µl of overnight bacteria culture and 100µl of eluted plaque (2.3.2.2) were mixed together in a new 1.5ml Eppendorf tube by vortexing. The BM25.8 and lambda phage mixture was incubated at 31°C for 30 minutes without shaking. Then, 400µl LB broth was added into the tube. And the tube was incubated at 31°C for an additional 1 hour with shaking at 225 rpm. After that, 1 – 10µl of infected cell suspension was spread on an LB/carbenicillin plate with a sterile glass spreader to obtain isolated colonies.

2.2.1.4 preparation of plasmid DNA

(Wizard® *Plus* SV Minipreps DNA Purification System)

Single, well-isolated bacterial colony containing recombinant plasmid was inoculated into 15 ml of LB medium with 100μg/ml ampicillin. The culture was incubated at 37°C for 16 hours with vigorous shaking. 10 ml of bacterial culture was collected by centrifugation for 15 minutes at 5000×*g*. The supernatant was then poured off and the tube was inverted to remove excess media on paper towel. The cell pellet was resuspended in 250μl of cell resuspension solution by vortexing. The cell suspension was then transferred to 1.5 ml Eppendorf tube. 250μl of cell lysis solution was then added into tube and mixed by gently inverting the tube several times to lyse the cells. 10μl of alkaline protease solution was added into the Eppendorf tube and mixed by gently invert the tube several times and then incubated at room temperature for 5 minutes. 350μl neutralization solution was added into the tube to neutralize the cell lysate with gently inversion. The neutralized lysate was centrifuged at 14,000×*g* for 30 minutes. Cleared lysate was carefully transferred to the spin column and centrifuged at 14,000×g for 1 minute. The flow-through was discarded. 750μl of column wash solution was added into the spin column and centrifuged at 14,000×g for 1 minute and the flow-through was discarded. The wash was repeated with 250μl column wash solution and centrifuged at 14,000×g for 2 minutes. The spin column was then transferred to a new Eppendorf tube and the plasmid DNA was eluted by adding 100μl of sterile dH_2O to spin column and centrifuged at 14,000×g for 1 minute. The quality of plasmid DNA was determined by measureing the optical density at 260nm and 280nm.

2.2.1.5 cycle DNA sequencing

(ABI Prism™ dRodamine Terminator Cycle Sequencing Ready Reaction kit)

The purified plasmid DNA was used as template for sequencing reaction by

using the ABI Prism™ dRodamine Terminator Cycle Sequencing Ready Reaction kit. The upper sequencing primer – MF656 were used for partial sequence of the cDNA insert in the λ TriplEx2 vector. 0.25μg of plasmid DNA was mixed with 4μl of terminator ready reaction mix, and 1.28μl of 1.25pmol/μl MF565 sequencing primer and made up to final volume 10μl with sterile dH_2O in PCR tube. The template DNA was denatured at 94°C for 2 minutes and then amplified for 25 cycles with a cycle protfile: 94°C for 15 seconds, 50°C for 30 seconds and 60°C for 4 minutes. The PCR product was purified by ethanol precipitation. The sequencing product was transferred into a 1.5 ml Eppendorf tube. 1μl of sodium acetate (pH4.6) and 25μl (2.5 volume) of 100% ethanol were added into the tube. The tube was mixed by vortex and placed on ice for 15 minutes to precipitate the extension product. The product was then centrifuged at 14,000×g for 30 minutes at 4°C. After discarding the supernatant, the pellet was rinsed with 125μl of 75% ethanol and centrifuged at 14,000×g for 15 minutes at 4°C. The supernatant was carefully removed as much as possible and the pellet were dried under vacuum. The dried pellet was then resuspended in 10μl of Hi-Di formide and heated at 95°C for 5 minutes. The sample was immediately chilled on ice and transferred to MicroAmp® Optical 96-well Reaction Plate, covering by MicroAmp® Strip Caps. The sample was loaded on ABI PRISM™ 3100 Genetic Analyzer for reading the nucleotide sequence. The sample was injected at 2.4kV for 30 seconds, and electrophoresis was run at 12.2kV at 42°C for 140 minutes. Raw data of the sequencing reaction were collected by ABI PRISM™ 3100 Genetic Analyzer Sequencing Analysis.

The nucleotide sequences were analyzed by the NCBI standard nucleotide – protein BLAST (BLASTX) in the Entrez Browser provided by the United State National Center for Biotechnology Information (www.ncbi.nlm.gov)

2.2.2. RT-PCR

2.2.2.1 Isolation of Total RNA by Guandidium Thiocyanate – Cesium Chloride ultracentrifugation.

The *S. japonicum* adult worm and cercaria were collected by Professor Zhongdao Wu, department of parasitology, medical school, Sun Yat-sen University, Guangzhou, China. The adult worms of *S. japonicum* were obtained form female rabbit infected 45 days with cercaria. The worm pairs were obtained by hepatic portal perfusion, washed extensively with PBS. The collected worm pairs were transferred into 10ml Guandidium Thiocyanate solution with 1% β-mercaptoethanol, and homogenized with hand-held homongenizer. The cercaria were collected in *Oncomelania hupensis* snail from JiangSu province(江苏省) under the induction of light. The collected cercaria were also transferred into Guandidium Thiocyanate solution with 1% β-mercaptoethanol, and homogenized with hand-held homongenizer. All of homogenized adult worm and cercaria were stored in -70°C until use.

The frozen cell lysate was thawed at 65°C water bath and the thawed cell lysate was further incubated at 65°C water bath for 5 more minutes. The thawed lysate was then chilled on ice immediately. To shear the genomic DNA molecules in the cell lysate, the thawed lysate was passed through a 20 g syringe needle for 50 times. After completely shearing, approximately 2ml of the cell lysate was layered on 1ml 5.7 M cesium chloride cushion in a DEPC treated ultracentrifuge polyallomer tube. The gradient was then centrifuged in a SW60 Ti rotor in a ultracentrifuge (Beckman) at 32,000 rpm for 18 hours at 18°C.

After centrifugation, the supernatant was removed by aspiration under vacuum. The tube was inverted quickly and drained for a while to remove excess supernatant and allow the RNA pellet to dry. Afterwards, the bottom 0.5 cm of the tube containing the clear RNA pellet was cut off with a new sterile scalpel blade. The RNA pellet was then rinsed out and resuspended very carefully to a new eppendorf tube with a total of 400µl DEPC treated ddH_2O. 45µl of 3M sodium acetate (pH4.8) and 1ml absolute ethanol was added to the RNA suspension. After mixing, the solution was centrifuged at maximum speed for 30 minutes at 4°C to obtain the RNA pellet. The RNA pellet was washed with 70% ethanol to remove any residual salts. The RNA pellet was vacuum dried and dissolved in 50µl of DEPC treated dH_2O and stored at -70°C until use

The yield of RNA was measured by absorbance at optical density 260 nm and 280 nm. An optical density of 1 unit corresponds to approximately 40 µg / ml for single stranded RNA. The ratio of the absorbance at 260 nm to 280 nm is a useful indication of purity. Pure preparation of RNA should give an $O.D._{260}$ / $O.D._{280}$ value of 2.0.

2.2.2.2 Synthesis of Fist Strand cDNA by reverse transcriptation reaction (RT)

1µg of total RNA in 10µl dH_2O was incubated at 65°C for 5 minutes and chilled on ice immediately. Then the RNA was added into a mixture of a total volume of 10µl containing 200 unit of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase, 0.2 mM of each dNTP, 0.1µg oligo $(dT)_{12\text{-}18}$, 1 x first-strand buffer (5 x, GibcoBRL), 10 mM dithiotheritol and 40 Unit RNaseOUT (GibcoBRL). The reaction mixture with final volume of 20µl was incubated at 37°C for 1 hour. The reaction

mixture was then diluted 10 folded with dH_2O after the RT reaction. The diluted samples were boiled for 5 minutes to denature the first strand DNA from the RNA template and then chilled on ice immediately for 2 minutes. The diluted samples were stored at -20°C until use.

2.2.2.3 PCR amplification of RT product

RT product of cercaria or worm RNA was used as templates for the PCR amplification. 40μl of mix containing 1 x reaction buffer IV, 0.2 mM of each dNTPs, 1 units of thermoprime^{PLUS} DNA polymerase, 1.5 mM $MgCl_2$ and 50 pmole each of specific primers were added into 10μl of boiled RT product containing first strand cDNA sample derived from 0.1μg total RNA. The template DNA was denatured at 94 °C for 3 minutes and then amplified for 30 cylces with a cycle profile: 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 1 minutes. After the last cycle, the reaction was extended at 72°C for 3 minutes. 10μl of amplified products were electrophoresis analyzed on agarose gel with 0.25 μg / ml ethidium bromide. After gel electrophoresis, the gel was visualized under UV illumination.

2.2.3 Rapid Amplification of cDNA Ends (RACE)

2.2.3.1 Synthesis of first strand cDNA for RACE reaction

SMART^{TM} RACE cDNA ampification kit (Clontech K1811-1) was used to obtain the 5' & 3' cDNA ends of the Sj-PIG-N gene.

To prepare the 5' – RACE – Ready cDNA, 1.5μg of total RNA of *Schistosoma japonicum* adult worm was mixed with 1μl of 5' – CDS primer and 1μl of SMART II

A oligo in a 1.5ml Eppendorf tube. Sterile dH_2O was added to the tube to a final volume of 5µl. The tube was incubated at 70°C for 2 minutes and chilled on ice immediately. A mixture containing 2µl of 5 x First – Strand buffer, 1µl of DTT (20 mM), 1µl of dNTPs (10mM) and 1µl of PowerScript Reverse Transcriptase was added into the tube. The contents of the tube was then mixed by gently pipetting and then spun for a few seconds. The tube was incubated at 42°C for 1.5 hours in an air incubator. And then the RT product was diluted with 100µl Tricine–EDTA Buffer. The diluted product was stored at -20°C until use.

To prepare the first strand cDNA for 3' RACE, 1.5µg of total RNA of *Schistosoma japonicum* adult worm was mixed with 1µl of 3' – CDS primer in a 1.5ml eppendorf tube. Sterile dH_2O was added to the tube to a final volume of 5µl. And follow the procedure as described in the preparation of the 5' – RACE – Ready cDNA. Finally, the RT product was also diluted with 100µl Tricine – EDTA Buffer and stored at -20°C until use.

2.2.3.2 5' RACE for Sj-PIG-N gene

For 5'RACE, 2.5µl of 5' RACE-Ready cDNA was used as the template. The template was added into the reaction mix containing 1µl of 50X Advantage 2 Polymerase Mix, 1µl of dNTP Mix (10 mM), 5µl of 10X Advantage 2 PCR Buffer, 5µl universal primer mix (UPM) and 1µl of Gene specific primers. The total volume of the reaction is 50µl. The PCR was performed by initiated denaturation at 94°C for 3 minutes and then amplified for 25 cycles with a cycle profile: 94°C for 30 seconds, 68° C for 30 seconds and 72°C for 3 minutes. After the last cycle, the reaction extend at 72 °C for 5 minutes. 10µl of amplified products were electrophoresed on agarose gel with

0.25μg/ml ethidium bromide. After gel electrophoresis, the gel was visualized under UV illumination.

2.2.3.3 3'RACE for Sj-PIG-N gene

For 3' RACE, 2.5 μl of 3' RACE-Ready cDNA was used as the template. The PCR reactions were conducted under same condition for 5'RACE PCR. The PCR was performed by initiated denaturation at 94°C for 3 minutes and then amplified for 25 cycles with a cycle profile: 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 3 minutes. After the last cycle, the reaction extend at 72°C for 5 minutes. 10μl of amplified products were electrophoresed on agarose gel with 0.25μg/ml ethidium bromide. After gel electrophoresis, the gel was visualized under UV illumination.

2.2.3.4 Purification of DNA fragment from agarose gel

The PCR products of 5' RACE and 3' RACE were analyzed by agarose gel electrophoresis. After gel electrophoresis, the DNA bands of corresponding sizes were excised from the gel by sterile blades under UV illumination and purified by QIAEX II Gel Extraction Kit. Gel excised was put into a new Eppendorf tube and 400μl of Buffer QX1was added to solubilize the agarose gel matrix. Then 10μl of reususpended QIAEX resin was added and incubated at 65°C for 10 minutes, with vortexing for every 2 minutes to keep QIAEX resin in suspension and thus enhancing DNA binding. The suspension was centrifuged at maximum speed for 30 seconds and the supernatant was discarded. The resin pellet was washed with 500μl QX1 Buffer and then washed twice with 500μl PE Buffer. Finally, the resin pellet was air-dried for 15 minutes to remove any volatile PE Buffer and then DNA was eluted by resuspending the pellet with 20μl

milli-Q water. It was centrifuged for 1 minute to pellet the resin. The eluted DNA was pipetted carefully into a sterile eppendorf tube.

2.2.3.5 Ligation of purified PCR fragments and pBluescript II KS(+) T-vector

6.5μl of RACE DNA fragments (insert, as prepared in 2.3.3.3 and 2.3.3.4) and 0.5μl of pBluescript II KS(+) T-vector were mixed together with 1μl of 10mM ATP, 1μl of 10X T4 DNA Ligase Buffer, and 7 Weiss unit of T4 DNA ligase (final volume of 10μl). The reaction mix was incubated at 16°C overnight.

2.2.3.6 Preparation of DH5α competent cells

Fresh DH5α bacterial colonies were obtained from glycerol stocks. Certain frozen bacteria-glycerol was scrapped by sterile inoculation loop and streaked on LB agar plates. The plates were incubated at 37°C overnight to allow development of bacterial colonies. Single colony was then picked up and inoculated into 15ml LB medium in 150ml flask. It was shaken at 250 rpm at 37°C for overnight as a starter culture. 1 ml of overnight culture was added to 100ml pre-warmed LB medium (1:100 dilution) in a 1-L E-flask and shaked at 250rpm, 37°C until the optical density of the culture at 550nm reached 0.2-0.4 (cell density around 4-9 x 10^7 cells/ ml). The culture was then collected and incubated on ice for 15 minutes. The bacterial cells were harvested by centrifugation at 1000 X g for 15 minutes at 4°C. The cell pellet was resuspended with 30ml pre-chilled RF1 solution. The cell suspension was allowed to incubate on ice for another 15 minutes before pelleted again with the same conditions. The resulting cell suspension was dispersed in 8ml pre-chilled RF2 and incubated for 15 minutes on ice. The resulting cell suspension was then aliquot into chilled 1.5ml

eppendorf with 150µl each. The tubes were flash freezed by liquid nitrogen and kept at –70°C freezer as stock of competent cells.

2.2.3.7 Transformation of recombinant plasmid

An aliquot of competent cell (150µl) was taken out from –70°C freezer and was thawed on ice. 10µl of ligation mix prepared from 2.3.5.4 was added and the ligation-competent cell mixture was kept on ice for 1 hours, then heat-shock was performed by putting the tube in 42°C water bath for 2 minutes and then chilled on ice immediately. 800µl of pre-warmed LB was added and it was shaked at 250rpm at 37° for an hour. Bacterial cells were harvested by short spin at high speed and finally resuspended with 200µl LB medium, afterwards spreaded on LB agar plates containing 100µg/ml of ampicillin, 800µg of X-gal and 800µg of IPTG. The plates were incubated at 37°C overnight and white colonies were identified as positive recombinants.

2.2.4 mammalian cell transfection

2.2.4.1 Stable transfection

About $2x10^5$ F9 PIG-N double knock-out cell line were seeded in a 60-nm tissue culture plate with in 4ml completed RPMI containing 10% serum. Cells were incubated in a CO_2 incubator until the cells were 30-50% confluent. The following solutions were prepared in 1.5ml eppendorf tubes:

Solution A: 2µg of pEGFP-Hyg was diluted in 1ml of Opti-MEM® I reduced serum medium.

Solution B: 4µl of DMIRE-C reagent were diluted into 1ml of Opti-MEM® I reduced serum medium.

The solution A and solution B were mixed gently and incubated at room temperature for 45 minutes. The cultured cells were washed once with 2ml serum free medium. After removed the serum free medium, the cells were overlaid with the lipid-DNA complex solution. The cells were incubated for 5 hours in a CO_2 incubator. After that, the DNA containing medium was replaced with 4ml of growth medium containing normal concentration of serum and the cells were incubated at 37°C in CO_2 incubator for 48hours. And then the cells were sub-cultured at ratio 1:5, into a new culture flask containing selection medium with 150µg/ml hygromycine B. The cells were cultured in selection medium for 2 weeks, and the selection medium were changed every 2 days.

2.2.4.2 Transient transfection

About $2x10^6$ 3T12 cells were seeded in a 35-mm tissue culture with in 2ml completed RPMI containing 10% serum. Cells were incubated in a CO_2 incubator until the cells were 60-80% confluent. The following solutions were prepared in 1.5ml eppendorf tubes:

Solution A: 2µg of pEGFP-Hyg was diluted in 500µl of Opti-MEM® I reduced serum medium.

Solution B: 4µl of DMIRE-C reagent were diluted into 500µl of Opti-MEM® I reduced serum medium.

The solution A and solution B were mixed gently and incubated at room temperature for 45 minutes. The cultured cells were washed once with 2ml serum free medium. After removed the serum free medium, the cells were overlaid with the

lipid-DNA complex solution. The cells were incubated for 5 hours in a CO_2 incubator. 1ml of growth medium containing twice the normal concentration of serum were added into the culture plate without removing the DNA-containing medium and the cells were incubated at 37°C in CO_2 incubator for 24 hours. And then the transfected cells were observed under fluorescent microscope and its image was captured.

2.2.5 Biological function studies of Sj-PIG-N gene

2.2.5.1 Flow Cytometry (FACS) analysis

As a powerful and feasible technique, flow cytometry was widely used for cell surface antigen analysis.

For FACS analysis, F9 cells, F9 PIG-N double knock-out cells and F9/KO/Hyg-Sj-PIG-N cells were used. The 3 kinds of cells were cultured in 10ml cell culture flask for 2 days at initial density of 1×10^5 cells/ml. for each of the cell type, two sets 3×10^5 cells were then collected in a 15ml falcon tube for and spun down at 150×g for 10 minutes. One of the two falcon cells of each cell type was used as a blank control that was not treated with antibody in the preparation procedure. The cell pellets were resuspended in 200µl of FACS medium (1× PBS with 0.1% sodium azide and 1% fetal bovin serum). To block the Fc receptor, rat IgG and mouse IgG were added into all of the falcons, and the final concentration of each IgG was 10µg/ml. The mixtures were incubated at 4°C for 30 minutes with occasionally shaking. The cells were washed with 5ml FACS medium at 150×g for 10 minutes. The pellets were resuspened then in 100µl FACS medium containing 2µg of rat anti mouse Thy1 (CD90.2) antibody or phycoerythrin-conjugated anti-CD24 M1/69 (BD Pharmingen). The cells then were

incubated at 4°C for 30 minutes with occasionally shaking. After 3 times washing with FACS medium, the cell pellets that stained with phycoerythrin-conjugated anti-CD24 M1/69 were resuspended in 600µl FACS fixation buffer and loaded for FACS analysis; however, cell pellets that stained with rat anti mouse Thy1 (CD90.2) antibody were resuspened with 100µl FACS medium containing 2µg of rabbit anti rat IgG conjugated with FITC. The resuspened cells were then incubated at 4°C for 30 minutes with occasionally shaking in dark. The cells were washed with FACS medium for three times at 150×g for 10 minutes. Finally the cell pellets were resuspended in 600µl FACS fixation buffer and loaded for FACS analysis.

2.2.5.2 In Vitro Mannose Labeling of Microsomes and Characterization of Glycolipids

In Vitro Mannose Labeling of Microsomes and Characterization of Glycolipids Microsomes were prepared from 5×10^6 cells pretreated with 5µg/ml tunicamycin for 2 h. For this, cells were lysed hypotonically in 20 mM HEPES/NaOH (pH 7.4) and homogenized (Hong, *et al.*, 1999). Supernatant after 10,000 × g centrifugation was spun at 100,000 × g for 1 h, and the pellet was resuspended in 150 µl of buffer A (50 mM HEPES/NaOH pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM 5' AMP, 10 µM palmitoyl CoA, 1 µg/ml tunicamycin and EDTA free protease inhibitor cocktail) and 2 µCi of GDP-[^{3}H]mannose (American Radiolabeled Chemicals, Inc., USA) for 1 h at 37 °C. A synthetic substrate, GlcN-PI(C8), a gift from Dr. Mark Lehrman, University of Texas, was dissolved in 0.03 % Triton X-100 and 200 ng was added to the reaction. Extracted lipids were treated with jack bean α-mannosidase (Sigma) in buffer B (0.1 M NaOAc pH 5.0, 0.1 % Na-taurodeoxycholate and 1mM $ZnCl_2$). Glycolipids were separated on Kiesel gel 60 (Merck, Darmstadt, Germany)

with a solvent system of chloroform : methanol : water (10:10:3) and detected by Fuji BAS1500 image analyzer (Fuji Film Co., Tokyo, Japan).

2.2.6 Intracellular localization assay

For the immuno-staining, about $5x10^4$ cells were seed in 24-well plate and incubated at 37°C CO_2 incubator for overnight. After overnight incubation, the old medium was discarded. The cells were washed three times with PBS. The cells were then fixed with 3% paraformaldehyde for 15 minutes. After that, the cells were washed three times with PBS. First antibody was then added into the plate and incubated for 2 hours. After washed three times with PBS, the cells were incubated with secondary antibody for 1 hour. At last, the cells were washed three times with PBS and analyzed under confocal laser scanning microscope.

For the confocal laser scanning microscope analysis, the fluorescence probe MitoTracker (M-7514) specific for mitochondria was purchased from Molecular Probes (Eugene, OR). The emission peak of it is at 517 nm. CLSM (Zeiss, Model LSM510) and the attached software were used for the signal analysis.

Chapter Three RESULTS

3.1 Random sequence analysis of cercaria EST from *S. japonicum* cercaria stage cDNA library

For the screening of *S. japonicum* cercaria cDNA library, the phage library was plated onto top agar. After that, 11 phage plaques were randomly selected and transferred into lysogenic bacteria, BM25.8. As a consequence, the lambda TriplEx was converted into pTriplEx plasmid. The plasmid DNA was purified and used for cycle sequence (referred to 2.2.1). The sequence result each gene was analyzed with NCBI standard nucleotide – protein BLAST (BlastX) in the Entrez Browser provided by the United State National Center for Biotechnology Information (www.ncbi.nlm.gov). The Blast search results were summarized and listed in table 2. The sequence results of clones of P02, P05, P06, P07, P08, P09 and N01, those carrying inserts, are showed in (3.1.1). Moreover, for each of them, the 4-5 genes having the highest scores in sequence alignment and the alignment results were showed in (3.1.2).

Table 2. Summary of Blast search results of the sequenced *S. japonicum* cercaria cDNA library clones

Clones	Putative Gene Functions	Remarks
P02	*Schistosoma mansoni* thioredoxin glutathione reductase	Fig. 12
P05	translation elongation factor 1-gamma	Fig. 13
P06	*Schistosoma japonicum* calcium-binding protein	Fig. 14
P07	*Lumbricus rubellus* 40S ribosomal protein S27	Fig. 15
P08	phosphatidylinositol glycan class N	Fig. 16
P09	elongation factor 2	Fig. 17
N01	Hypothetical gene	Fig. 18
P01	---	Failed in cloning
P03	---	Failed in cloning
P04	---	Failed in cloning
P10	---	Failed in cloning

3.1.1 Sequencing results

```
1   GGTTACGTAT CTTAATGCTC GTGGGATGCT ATTAAGTCCT CATGAGGTTC
51  AGATTACAGA AAAGAATAAA AAAGTATCCA CAATAATTGG AAATAAAATC
101 ATCTTAGCTA CTGGCGAGCG TCCAAAATAC CCAGAAATAC CTGGAGCAAT
151 CGAATATGGG ATTACAAGTG ATGATTTGTT TTCCTTACCA TACTTCCCGG
201 GCAAAACACT GGTCGTTGGA GCGAGCTATG TTGCATTGGA ATGTGCTGGT
251 TTTCTTGCCA GTTTGGGCGG TGATGTTACT GTTATGGTTC GTTCCATTTT
301 GCTTCGTGGT TTCGATCAAC AAATGGCTGA GAAGGTTGGC GACTATATGG
351 AAAATCATGG AGTCAAGTTC GCAAAGTTGT GTGTACCAGA CGAGATTACA
401 CAGTTGAAAC CGGTAGATAC TGAGAATAAC AAACCTGGAC TCCTGCTTGT
451 TAAGGGTCAT TATACTGATG GTAAGAAGTT TGAAGAAGAA TTTGAAACGG
501 TCATTTTCGC TGTTGGTCGT GAACCACAAT TATCGAAGCT TAATTGTGAA
551 TGTCGGTGTT AAACTAAATA AGAATGGTCN GGTTGTATGC TCAGATGATG
601 CAAACTACAG TCAGTAACAT TTATGCCATT GGGGATANAA CGCTGGGAAA
651 CACAGTTAAC TCCAGTGGGC TATTCATGCT GGACGTTATT TGGCTAAACG
701 GTTATTCCCT GGNGCACTGA ACTGACTGGA CTATTCC
```

Fig. 5. Nucleotide sequence of P02 (737bp)

```
1   CATCTTTGGA GAGGATAAGA AAAACACAGT TAGTGGTCTT TGGATTTGGC
51  GAGGCACCGG TCTTATATTC GATTTGGATG AAGACCTACG AATAGATTAT
101 GTCTTACAGC TGGAGGAAAC TTGATCCCAA TTCAGAGGAA ACCAAAAGCC
151 TGTACATGAC TACTTTACGC GAAAATTCCA TGATAAACCA TTCAATCAAG
201 AAAATATTTA AATGAGAAAT AAAGGCTTCT GCTTAGTTGA AAAAAAAAAT
251 AAAAAAAAAA AAAAAAAAAA AAAAAAAAA
```

Fig. 6. Nucleotide sequence of P05 (279)

```
1   GATTTTTTCT AAGGTAAGTC TTTGTTGACT TGCTTGTCAA TTTAATAATT
51  ATCCTAGTGT TTTACGTATT GAGTAAAAAT GGTCAATCGA GCAGAGGCTG
101 CAACTTCTAA AACATCTTGA CAAAGATAAC AGTGGGAAAA TCAGCACACA
151 AGAGCTAATG GAATTTTTGA GCAGCGTTAA CTGCCCATTC AACAAAGCAC
201 AGGTTGAGAA GTTTATTAAA GTACATGACG CGGATGGAGA TGGACAACTA
251 AATACTGATG AACTTCTTAA AGTTTTGTGC CAATAATTTC TTGAATTTTC
301 AGTAATGTTA CTCTCATAAA TAAATACTAT TTTAATTTCA TAACACTGAA
351 AAAAAAAAAA AAAAAAAAAA AAAAA
```

Fig. 7. Nucleotide sequence of P06 (365bp)

```
1   GACATTTGGT TGCCGAAAAC GCGATTAGCA TGCCGCTTGC TCGTGATTTA
51  TTACACCCAA CTTTTCGAGA GGAGAAACGA AAGTGCAAAT TAAAACGTCT
101 GGTCCCCTCT CCAAACTCCT TTTTCATGGA TGTCAGGTGT TCAGGCTGTT
151 TGAAAATCCA AATCGTTTTC AGTCATGCGC AAACAGCTGT AGTTTGTCCG
201 GGATGTGACC GGGTATTGTG TCAGCCCACC GGTGGTAGAG CGCGATTAGC
251 TGATGGATGC AGCTACCGTA AGAAGACGAG ATAAAGTTTG GTAAATTCTG
301 ATAGAAACAA TAAATTTGTG CAAAAAAAAA AAAAAAAAAA AAAAAAAAAA
351 A
```

Fig. 8. Nucleotide sequence of P07 (351bp)

```
1   GATAGCACAG CGGGAATTCA TCCTGGAGAG ATGTCCTCTT ACACCGTGGT
51  GTATGGGGTG TGTCACATAC GAGAGTCCCT ACCGAATCAA GGCCTGCACA
101 TGTAGCTATA CTGGGAGGAT TCTATGAAGA TGTAGCCTCT ATAACTAATG
151 GGTGGAGGAC GAATCCTGTG GAGTTTGATA CCGTACTGAA TCGTTCTACC
201 CTAGCTTGGA TATGGGGTTA CAAGGAAGTT GTCATGTCCT TTGTACCACC
251 TTTTACACAT CACATCAAAG CAACTCCTTA CCCAGATGAA TTAAGTGATT
301 TGGCTAAAAC TAATCCTACA GAAATCGACC GATGGGTCAC CGATCAGTTT
351 ACGGACTTCA TCGATAATTC GGATGACTTC TTTGATGACC TTACTGCAAT
401 ATCTAATGAT TACCGTCAGG GTAGAATGGT ATTTCTGCAT TTAGATGCTG
451 CTGATATGGT TGGTCATTCT TTTAAACCTG ATTCACCTGA ATATGTTAAA
501 GTTCTTCGTA ATTTGGACAA GGTGATTTTC CGTGTTTATC ANAAGCTAAC
551 TGAAAAATCT CGTGGNAGTG ATTCACGTAT CGCTTATATC CTGACTTCTG
601 ATCATGGAAT GACTGAGTGG GGAAGTCATG GATCTGGNTC NTTGCATGAA
651 AAAATNAAAC CTTTGNTAAC CTTGGGGGTC TGGGAATGTC CGTCCCGTTN
701 AAATANAAAC TAAATATGAA AAACTTGTCC NAAAAANAAA AAAAA
```

Fig. 9. Nucleotide sequence of clone P08 (745bp)

```
1   CACAATCGTT TATATATGCG TGCTTCCCCA TTATCAGAGG ATTTAGCTGC
51  TGATATTGAC AGTGGAAAGA TCAATGCACA CCAAGACTCG AAAGAGCTTG
101 GTCGTTACTT AGCTGACAAT CATGGATGGG ATGTTCATGA AGGCAGAAAG
151 ATATGGTGCT TTGGTCCCGA AAACACGGGA CCTAATATTG TTGTAGATAC
201 CACTAAAGCA GTACAATACT TAAATGAGAT AAAAGATAGT ATTGTTAGTG
251 CATTCCAGTG GGTAACCAAA GACGGTGTTC TCTGTTCCGA AAATATGCGT
301 GGTGTTCGTA TCAATCTGGA AGACGTGACA TTACACGCAG ATGCCATCCA
351 TCGCGGAGGT GGCCAAATCA TAGGTACGGC ACGTCGATGC TTCTACGCCT
401 CTGTTCTTAC CGCCAAACCA GGGTTACTAG AACCAGTCTA TTTGGTAGAA
451 ATTCAAGGTC CGTCTAACAT TATGGGTGGT ATCTACAGCA CGTTGAACCG
501 CAAAAGAGCC GTTGTAGTTT CCGAGGAGCA NAAAGATGAC AGTCCAATGT
551 GTATTATCAA GGCGCACCTA CCCGTCAATG AATCTTTTGG NTTCACTACC
601 GATCTCGTGC TGCAACTGGT GGTCAGGCTT TCANCAGTGT TCCTCNATCA
651 CNGGCAATTT ATCAAGGGAT CCTATCGANC CGAATTCAAA ACTGGGCAGG
701 TANTTTTGGG GAAACCAAAA CGTAANGGTT AAT
```

Fig. 10. Nucleotide sequence of clone P09 (733bp)

```
1   TAATAAATAG GTTATAATCA TTATATTATC ATCTAAGTAA TACTTCACTT
51  AGTGATTAAT TATTTTAATG ATTATTTAAA AAAAGAGTTT TTTTTTCTTT
101 TTCTTGTTTC TTCTCAGTTC ATTTAAGAGT AAGCGTTAGT TTACATCAAA
151 AACAAAAAAA CAAAAAAAAA GAAAACTGTA ATCAAATTAA CAAATTTATG
201 TAGTTTGTGA ACTTTGATAA TTTAATTTTG TGCATACTTC AGTCAATCGA
251 GTAGTTGACA TTATCATTAT CATTAATAAN TGAAACGTGT CTATATTAAA
301 GGCATGATTA TGCAATGGCT TGATGTCTTT AATATTTAGG TATACAATAA
351 AATGAATAGA ATGTTAGTAA TATCAGTGAT AGTAGAAAAG GAATTAAATA
401 TGGAGAATAT AATTGANAAA AAAAAAA
```

Fig. 11. Nucleotide sequence of clone N01 (427bp)

3.1.2 BlastX Search results

The sequences of clone P02, P05, P06, P07, P08, P09, and N01 were applied in the BlastX search. The results of BlastX search were showed below.

```
                                                                    Score    E
Sequences producing significant alignments:                        (bits) Value
gi|15149312|gb|AAK85233.1|AF395822_1  thioredoxin glutathion...     348   e-100
gi|29825894|gb|AAN63051.1|  thioredoxin glutathione reductas...     207   7e-53
gi|29825896|gb|AAN63052.1|  thioredoxin glutathione reductas...     207   7e-53
gi|32451906|gb|AAH54599.1|  Unknown (protein for MGC:64035) ...     188   2e-48
gi|15826812|pdb|1H6V|A  Chain A, Mammalian Thioredoxin Reduc...     186   2e-47
```

gi|15149312|gb|AAK85233.1|AF395822_1 thioredoxin glutathione reductase [Schistosoma mansoni]
Length = 598. Score = 348 bits (894), Expect (2) = e-100
Identities = 171/183 (93%), Positives = 178/183 (97%) Frame = +2

```
Query: 2   VTYLNARGMLLSPHEVQITEKNKKVSTIIGNKIILATGERPKYPEIPGAIEYGITSDDLF 181
           VTYLNA+G L+SPHEVQIT+KN+KVSTI GNKIILATGERPKYPEIPGA+EYGITSDDLF
Sbjct: 221 VTYLNAKGRLISPHEVQITDKNQKVSTITGNKIILATGERPKYPEIPGAVEYGITSDDLF 280

Query: 182 SLPYFPGKTLVVGASYVALECAGFLASLGGDVTVMVRSILLRGFDQQMAEKVGDYMENHG 361
           SLPYFPGKTLV+GASYVALECAGFLASLGGDVTVMVRSILLRGFDQQMAEKVGDYMENHG
Sbjct: 281 SLPYFPGKTLVIGASYVALECAGFLASLGGDVTVMVRSILLRGFDQQMAEKVGDYMENHG 340

Query: 362 VKFAKLCVPDEITQLKPVDTENNKPGLLLVKGHYTDGKKFEEEFETVIFAVGREPQLSKL 541
           VKFAKLCVPDEI QLK VDTENNKPGLLLVKGHYTDGKKFEEEFETVIFAVGREPQLSK+
Sbjct: 341 VKFAKLCVPDEIKQLKVVDTENNKPGLLLVKGHYTDGKKFEEEFETVIFAVGREPQLSKV 400

Query: 542 NCE 550
           CE
Sbjct: 401 LCE 403
```

Fig. 12. BLASTX window showing the search results of P02. Upper part shows the 5 genes having the highest scores in sequenc alignment. The lower part shows the alignment results of P02 to the amino acid sequence of the thioredoxin glutathione reductase in *Schistosoma mansoni*

Sequences producing significant alignments:	Score (bits)	E Value
gi|119164|sp|P12261|EF1G_ARTSA Elongation factor 1-gamma (E...	47	1e-08
gi|18874391|gb|AAL78751.1| translation elongation factor-1 ...	47	1e-08
gi|232037|sp|P29694|EF1G_RABIT Elongation factor 1-gamma (E...	38	5e-07
gi|27661344|ref|XP_215165.1| similar to Elongation factor 1...	38	5e-07
gi|30584897|gb|AAP36704.1| Homo sapiens eukaryotic translat...	37	6e-07

gi|119164|sp|P12261|EF1G_ARTSA Elongation factor 1-gamma (EF-1-gamma)(eEF-1B gamma)
gi|84607|pir||S00162 translation elongation factor eEF-1 gamma chain - brine shrimp
gi|161172|gb|AAC83401.1| elongation factor 1-gamma [Artemia sp.]
Length = 430 Score = 46.6 bits (109), Expect(2) = 1e-08.
Identities = 17/33 (51%), Positives = 24/33 (72%) Frame = +2

```
Query: 2   IFGEDKKNTVSGLWIWRGTGLIFDLDEDLRIDY 100
           +FGED   +++SG+W+WRG  L F L   D +IDY
Sbjct: 356 VFGEDNDSSISGIWVWRGQDLAFKLSPDWQIDY 388
```

gi|18874391|gb|AAL78751.1| translation elongation factor-1 gamma [Locusta migratoria]
Length = 438. Score = 46.6 bits (109), Expect(2) = 1e-08.
Identities = 16/33 (48%), Positives = 24/33 (72%) Frame = +2

```
Query: 2   IFGEDKKNTVSGLWIWRGTGLIFDLDEDLRIDY 100
           +FG D  +++SG+W+WRG  L FDL   D ++DY
Sbjct: 364 LFGSDNDSSISGIWVWRGQDLAFDLSPDWQVDY 396
```

Fig. 13. BLASTX window showing the search results of P05. Upper part shows the 5 genes having the highest scores in sequence alignment. The lower part shows the alignment results of P05 to the amino acid sequence of the translation elongation factor in Artemia, *Locusta migratoria*.

Sequences producing significant alignments:	Score (bits)	E Value
gi|29650916|gb|AAO86062.1| 8 kDa calcium-binding protein [S...	126	8e-29
gi|115391|sp|P13566|CABP_SCHMA CALCIUM-BINDING PROTEIN (CAB...	105	1e-22
gi|22655518|gb|AAN04091.1|AF527456_1 calcium binding protei...	63	1e-09
gi|6911186|gb|AAF31420.1|AF213970_1 putative calcium-bindin...	55	3e-07

gi|29650916|gb|AAO86062.1| 8 kDa calcium-binding protein [*Schistosoma japonicum*]
gi|30523352|gb|AAP31909.1| 8 kDa calcium-binding protein [*Schistosoma japonicum*]
Length = 69. Score = 126 bits (316), Expect = 8e-29
Identities = 61/61 (100%), Positives = 61/61 (100%). Frame = +2

```
Query: 101 QLLKHLDKDNSGKISTQELMEFLSSVNCPFNKAQVEKFIKVHDADGDGQLNTDELLKVLCQ283
           QLLKHLDKDNSGKISTQELMEFLSSVNCPFNKAQVEKFIKVHDADGDGQLNTDELLKVLCQ
Sbjct: 9   QLLKHLDKDNSGKISTQELMEFLSSVNCPFNKAQVEKFIKVHDADGDGQLNTDELLKVLCQ 69
```

gi|115391|sp|P13566|	CABP_SCHMA CALCIUM-BINDING PROTEIN (CABP)
gi|84404|pir||A30792	calcium-binding protein - fluke (Schistosoma mansoni)
gi|160939|gb|AAA29860.1|	calcium-binding protein
gi|160941|gb|AAA29861.1|	calcium binding protein

Length = 69. Score = 105 bits (262), Expect = 1e-22
Identities = 50/60 (83%), Positives = 53/60 (88%). Frame = +2

```
Query: 101 QLLKHLDKDNSGKISTQELMEFLSSVNCPFNKAQVEKFIKVHDADGDGQLNTDELLKVLC 280
           QLLKHLD+D SGKIS+QELMEFL +VNCPF K QVEKFIK HD DGDGQLNTDELL VLC
Sbjct: 9   QLLKHLDRDKSGKISSQELMEFLHTVNCPFKKEQVEKFIKQHDKDGDGQLNTDELLDVLC 68
```

Fig. 14. BLASTX window showing the search results of P06. Upper part shows the 4 genes having the highest scores in sequence alignment. The lower part shows the alignment results of P06 to the amino acid sequence of the calcium-binding protein in *S. japonicum*, and *S. mansoni*.

```
                                                                  Score    E
Sequences producing significant alignments:                       (bits) Value
gi|6066480|emb|CAB58439.1|  40S ribosomal protein S27 [Lumbr...   114   4e-25
gi|13277528|gb|AAH03667.1|AAH03667  Similar to 40S ribosomal...   112   1e-24
gi|7705706|ref|NP_057004.1|  ribosomal protein S27-like prot...   112   1e-24
gi|15294067|gb|AAK95210.1|AF402836_1  40S ribosomal protein ...   112   1e-24
gi|1350972|sp|P47904|RS27_XENLA  40S ribosomal protein S27 >...   111   2e-24
gi|15294069|gb|AAK95211.1|AF402837_1  40S ribosomal protein ...   111   2e-24
```

gi|6066480|emb|CAB58439.1| 40S ribosomal protein S27 [Lumbricus rubellus

Length = 84. Score = 114 bits (284), Expect = 4e-25

Identities = 52/82 (63%), Positives = 59/82 (71%). Frame = +3

```
Query: 30  MPLARDLLHPTFXXXXXXXXXXXXVPSPNSFFMDVRCSGCLKIQIVFSHAQTAVVCPGCD 209
           MPL RDLLHPT             V SPNSFFMDV+C GC KI   VFSHAQT V+C GC+
Sbjct: 1   MPLTRDLLHPTLKDEKRKCKLKRLVQSPNSFFMDVKCPGCYKITTVFSHAQTVVLCVGCN 60

Query: 210 RVLCQPTGGRARLADGCSYRKK 275
            VLCQPTGG+ARL +GCS+R+K
Sbjct: 61  TVLCQPTGGKARLTEGCSFRRK 82
```

gi|13277528|gb|AAH03667.1|AAH03667 Similar to 40S ribosomal protein S27 [Homo sapiens].

Length = 84. Score = 112 bits (281), Expect = 1e-24

Identities = 51/82 (62%), Positives = 59/82 (71%). Frame = +3

```
Query: 30  MPLARDLLHPTFXXXXXXXXXXXXVPSPNSFFMDVRCSGCLKIQIVFSHAQTAVVCPGCD 209
           MPLARDLLHP+             V SPNS+FMDV+C  GC KI  VFSHAQT V+C GC
Sbjct: 1   MPLARDLLHPSLEEEKKKHKEKRLVQSPNSYFMDVKCPGCYKITTVFSHAQTVVLCVGCS 60

Query: 210 RVLCQPTGGRARLADGCSYRKK 275
            VLCQPTGG+ARL +GCS+R+K
Sbjct: 61   TVLCQPTGGKARLTEGCSFRRK 82
```

Fig. 15. BLASTX window showing the search results of P07. Upper part shows the 6 genes having the highest scores in sequence alignment. The lower part shows the alignment results of P07 to the amino acid sequence of the 40S ribosomal protein S27 in *Lumbricus rubellus* and *Homo sapiens.*

Sequences producing significant alignments:	Score (bits)	E Value
gi|5631310|dbj|BAA82620.1| phosphatidylinositolglycan class...	170	2e-41
gi|7305383|ref|NP_038812.1| phosphatidylinositol glycan, cl...	170	2e-41
gi|18088159|gb|AAH21148.1| Pign protein [Mus musculus]	170	2e-41
gi|5668571|dbj|BAA82663.1| phosphatidylinositolglycan class...	170	2e-41
gi|20306653|gb|AAH28363.1| Phosphatidylinositol glycan, cla...	166	3e-40
gi|6912500|ref|NP_036459.1| phosphatidylinositol glycan, cl...	166	3e-40

gi|7305383|ref|NP_038812.1| phosphatidylinositol glycan, class N [Mus musculus]

gi|5631308|dbj|BAA82619.1| phosphatidylinositolglycan class N [Mus musculus].

Length = 931. Score = 170 bits (431), Expect = 2e-41

Identities = 83/218 (38%), Positives = 121/218 (55%), Gaps = 1/218 (0%). Frame = +3

```
Query: 27  RDVLLHRGVWGVSHTRVPTESRPAHVAILGGFYEDVASITNGWRTNPVEFDTVLNRSTLA 206
           R+V++H G WGVSHTRVPTESRP  HVA++ GFYEDV+++  GW+ NPVEFD++ N S
Sbjct: 74  RNVIMHEGSWGVSHTRVPTESRPGHVALIAGFYEDVSAVAKGWKENPVEFDSLFNESKYT 133

Query: 207 WIWGYKEVVMSFVPPFT-HHIKATPYPDELSDLAKTNPTEIDRWVTDQXXXXXXXXXXXX 383
            W WG  +++  F    +  H+     Y  +   D    +  T++D WV  D+
Sbjct: 134 WSWGSPDILPMFAKGASGDHVYTYSYDAQREDFGAHDATKLDTWVFDKVKDFFDAARNNQ 193

Query: 384 XXLTAISNDYRQGRMVFLHLDAADMVGHSFKPDSPEYVKVLRNLDKVIFRVYXKLTEKSR 563
              T ++    +  +  FLHL    D  GH+ +P S EY    ++ +D  +  +   + +
Sbjct: 194 SLFTKVN---EEKVVFFLHLLGVDTNGHAHRPSSREYKDNIKKVDDGVKEI-VSIFKHFY 249

Query: 564 GSDSRIAYILTSDHGMTEWGSHGSGSLHEKXKPLXTLG 677
           G D + A+I TSDHGMT+WGSHG+G    E    P  T G
Sbjct: 250 GDDGKTAFIFTSDHGMTDWGSHGAGHPSETLTPFVTWG 287
```

Fig. 16. BLASTX window showing the search results of P08. Upper part shows the 6 genes having the highest scores in sequence alignment. The lower part shows the alignment results of P08 to the amino acid sequence of phosphatidylinositol glycan, class N in *Mus musculus*

Sequences producing significant alignments:	Score (bits)	E Value
gi|19353009|gb|AAH24689.1| Similar to Elongation factor 2b ...	287	1e-76
gi|181969|gb|AAA50388.1| elongation factor 2	287	1e-76
gi|4503483|ref|NP_001952.1| eukaryotic translation elongati...	287	1e-76
i|2494246|sp|Q90705|EF2_CHICK Elongation factor 2 (EF-2) >...	286	2e-76
gi|12805513|gb|AAH02233.1| Eef2 protein [Mus musculus]	286	3e-76
gi|2130649|gb|AAD05363.1| EF-2 [Rattus norvegicus]	286	3e-76

gi|19353009|gb|AAH24689.1| Similar to Elongation factor 2b [Homo sapiens]

Length = 517. Score = 287 bits (734), Expect = 1e-76.

Identities = 133/201 (66%), Positives = 158/201 (78%). Frame = +1

```
Query: 1   HNRLYMRASPLSEDLAADIDSGKINAHQDSKELGRYLADNHGWDVHEGRKIWCFGPENTG 180
           HNRLYM+A  P  + LA DID G+++A Q+ K+   RYLA+ + WDV E RKIWCFGP+ TG
Sbjct: 258 HNRLYMKARPFPDGLAEDIDKGEVSARQELKQRARYLAEKYEWDVAEARKIWCFGPDGTG 317

Query: 181 PNIVVDTTKAVQYLNEIKDSIVSAFQWVTKDGVLCSENMRGVRINLEDVTLHADAIHRGG 360
           PNI+ D TK VQYLNEIKDS+V+ FQW TK+G LC ENMRGVR ++ DVTLHADAIHRGG
Sbjct: 318 PNILTDITKGVQYLNEIKDSVVAGFQWATKEGALCEENMRGVRFDVHDVTLHADAIHRGG 377

Query: 361 GQIIGTARRCFYASVLTAKPGLLEPVYLVEIQGPSNIMGGIYSTLNRKRAVVVSEEXKDD 540
           GQII TARRC YASVLTA+P L+EP+YLVEIQ P  ++GGIY  LNRKR  V  E
Sbjct: 378 GQIIPTARRCLYASVLTAQPRLMEPIYLVEIQCPEQVVGGIYGVLNRKRGHVFEESQVAG 437

Query: 541 SPMCIIKAHLPVNESFGFTTD 603
           +PM ++KA+LPVNESFGFT D
Sbjct: 438 TPMFVVKAYLPVNESFGFTAD 458
```

Fig. 17. BLASTX window showing the search results of P09. Upper part shows the 6 genes having the highest scores in sequence alignment. The lower part shows the alignment results of P09 to the amino acid sequence of elongation factor 2b in *Homo sapiens*.

```
                                                                  Score    E
Sequences producing significant alignments:                      (bits) Value
gi|17046925|gb|AAL34925.1|  envelope glycoprotein [Human imm...   30   9.6
```

gi|17046925|gb|AAL34925.1| envelope glycoprotein [Human immunodeficiency virus type 1]

Length = 613. Score = 29.6 bits (65), Expect = 9.6

Identities = 13/40 (32%), Positives = 23/40 (57%). Frame = -2

```
Query: 336 NIKDIKPLHNHAFNIDTFXLLMIMIMSTTRLTEVCTKLNY 217
           NI DI PL+N   N   F    ++    +T+ +T+ C K+++
Sbjct: 167 NILDIVPLNNENNNTKNFSDYRLISCNTSTITQACPKVSW 206
```

Fig. 18. BLASTX window showing the search results of N01. Upper part shows the one gene having the highest scores in sequence alignment. The lower part shows the alignment results of N01 to the amino acid sequence of envelope glycoprotein of Human immunodeficiency virus type 1.

3.2 The expression of Sj-PIG-N gene in both adult worms and cercaria

3.2.1 Spectrophotometric analysis of total RNA

Total RNAs of *S. japonicum* adult worms and were isolated by the guanidium thiocyanate cesium chloride method (Chirgwin, *et al.,* 1979) (referred to 2.2.2.1). Absorbance of UV light at 260 nm and 280 nm were measured to analyze the yield and purity of the RNA samples (table 3). The ratio of absorbance at 260 nm to absorbance at 280 nm among the samples was roughly around 1.5. The amount of RNA obtained was measured by absorbance at 260 nm. An optical density of 1 unit corresponds to approximately 40 ug / ml for single stranded RNA. The RNAs were electrophoresed in a 1% agarose gel stained with ethidium bromide (Fig. 19).

Table 3. Spectrophotometric analysis of RNA samples isolated from *S. japonicum* adult worms and cercaria.

	S. japonicum adult worms	*S. japonicum* cercaria
OD_{260}/OD_{280}	1.59	1.57
Conc. (μg/μl)	0.584	0.324
Yield (μg)	32.7	18.144

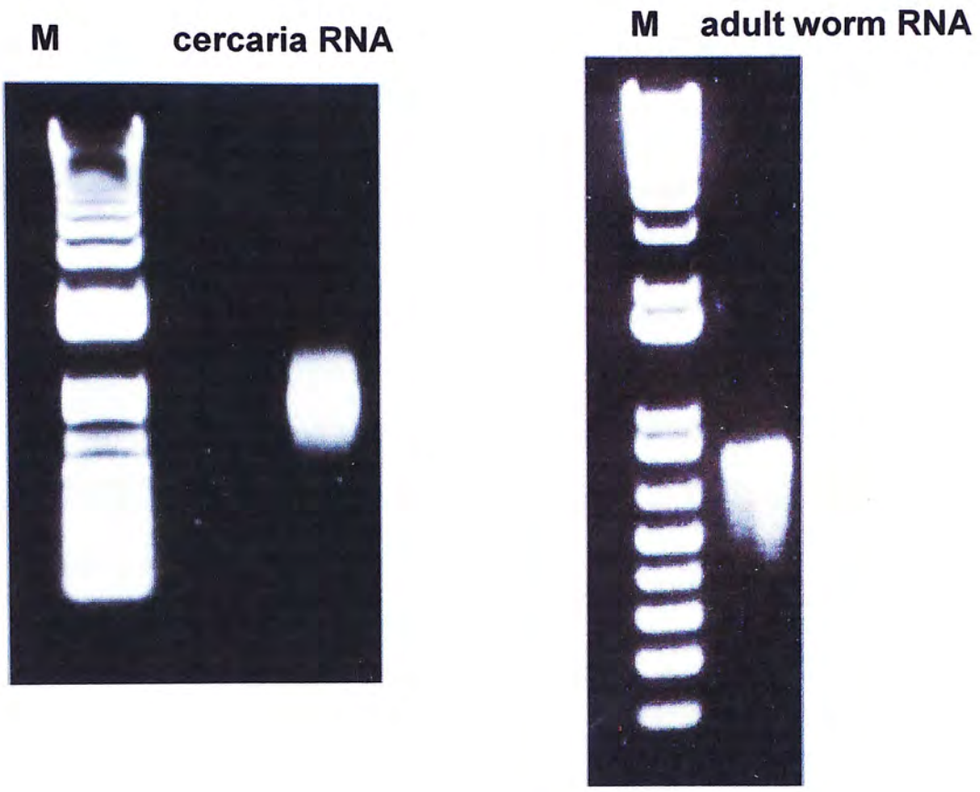


Fig. 19. RNA samples of *S. japonicum* adult worms and cercaria. 0.5μg of adult worms RNA and 0.38μg of cercaria RNA were loaded on a 1% agarose gel and electrophoresed. M = 1 kb plus DNA size marker.

3.2.2 Detection of Sj-PIG-N gene expression in both adult worm and cercaria stages

The translated amino acids sequence of Clone P8 is homologous to that of phosphatidylinositol glycan, class N in *Mus musculus* and human. In mammalian cells, the PIG-N enzyme is involved in the GPI anchor synthesis. The function of PIG is to transfer the EtN-P to the first mannose on GPI anchor main core structure. Until now, there is no GPI anchor synthesis related enzyme that has been reported in schistosome. The clone P8, which is called *S. japonicum* PIG-N, was further analyzed to characterize its identity.

The gene was cloned from *S. japonicum* cercaria cDNA library. Being a GPI anchor synthesis enzyme, it is reasonable to believe that it expressed in both adult worm stage and cercaria stage. For the detection of Sj-PIG-N expression in both adult worms and cercaria, the Sj-PIG-N gene was amplified from total RNAs of adult worms and cercaria by RT-PCR using gene specific primers – MF802 and MF804 as described in (2.2.2). The resulting PCR products were 860bp. The PCR products were electrophoresed in a 1% agarose gel stained with ethidium bromide (Fig. 20).

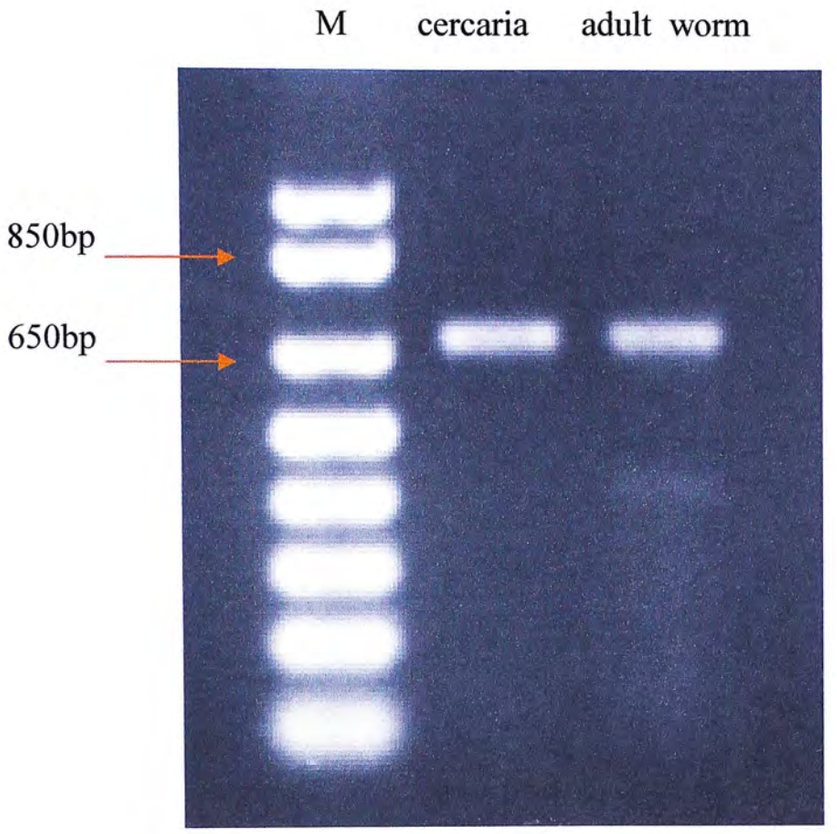


Fig. 20. Detection of Sj-PIG-N gene expression in both adult worm and cercaria by RT-PCR. The Sj-PIG-N gene transcripts were amplified with MF802 and MF804 for 30 cycles in equal volume of cDNAs derived from 0.1 μg total RNAs of adult worms and cercaria. The template DNA was denatured at 94°C for 3 minutes and then amplified for 30 cylces with a cycle profile: 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 1 minutes. After the last cycle, the reaction extend at 72°C for 3 minutes. 10μl of PCR products were loaded on a 2% agarose gel and electrophoresed. M = 1 kb plus DNA size marker.

3.3 Cloning of the full length of Sj-PIG-N gene

3.3.1 Amplification of the full length of Sj-PIG-N gene

For obtaining the full length sequence of Sj-PIG-N gene, SMART^{TM} RACE cDNA ampification kit (Clontech) was used to clone the 5' & 3' cDNA ends. Based on the sequence data of clone P8, gene sepecific primers (GSP) for 5' & 3 Rapid Amplification cDNA Ends (referred to 2.2.3) were designed by Olig6 program. All GSPs are 22-27 nt long with a Tm at least 80°C and have a GC content of 50-70%.

Followed the protocol of SMART^{TM} RACE cDNA ampification kit (Clontech) which was described in (2.2.3). 1.5μg of total RNA of Schistosoma japonica adult worm were used for first strand cDNA synthesis. The 5' end fragments were amplified with universal primer mix (UPM) and GSP – MF804. The 3' end fragments were amplified with UPM and GSP – MF802 and nested GSP MF803. The PCR products of 5' and 3' RACE PCR were purified from agarose gel and ligated into pBluescriptII KS(+) T vector. After that, the gene fragments were sequenced with cycle sequencing. Gene sepecific primers for sequencing and amplifying the full length of Sj-PIG-N gene were qdesigned based on 5' and 3' RACE results. All squencing primers are 22-27 nt long with a Tm at least 65°C and have a GC content of 50-70%.

To amplify the full length Sj-PIG-N gene, RT product containing first strand cDNA sample derived from 0.1μg total RNA (referred to 2.2.2) were added into the PCR reaction mix containing 1x Advantage II DNA polymerase, 1x Advantage II reaction buffer and 50pmole of gene specific primers – MF834 and MF838. The total

volume of reaction mix is 15µl. The PCR products were electrophoresed in a 1% agarose gel stained with ethidium bromide (Fig. 21). The resulting PCR product was 3.0 kb.

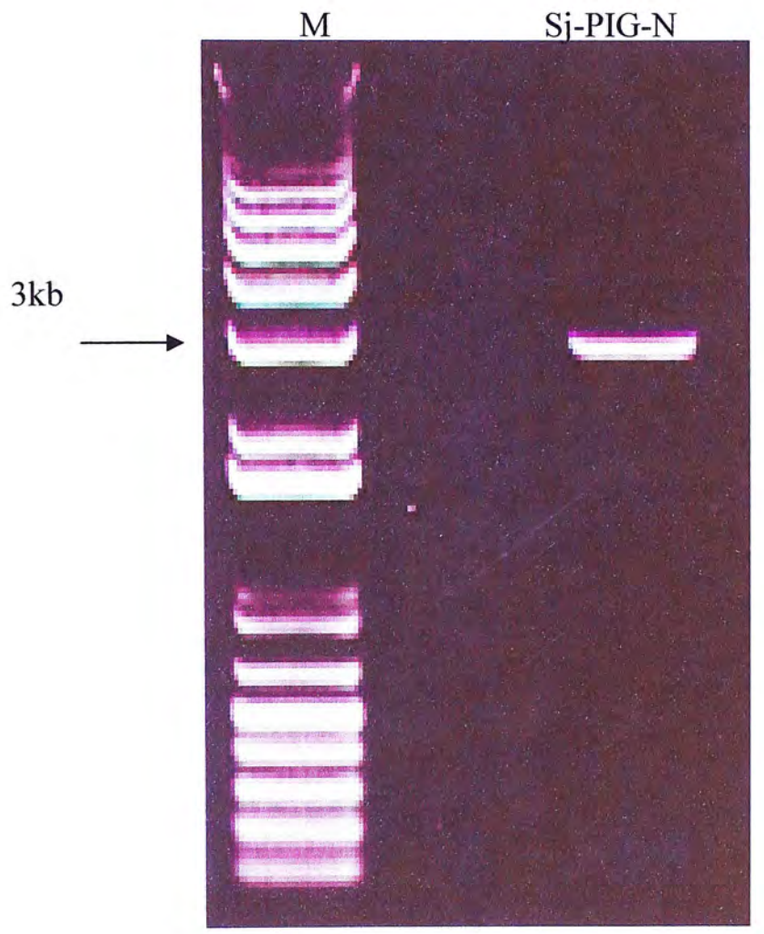


Fig. 21. Amplification of full length of Sj-PIG-N gene. The Sj-PIG-N gene was amplified with gene specific primers – MF834 and 838. The resulting PCR product was about 3.0kb. The total volume of reaction mix is 15µl. The template DNA was denatured at 94°C for 3 minutes and then amplified for 35 cycles with a cycle profile: 94°C for 30 seconds, 64°C for 30 seconds and 72°C for 3.5 minutes. After the last cycle, the reaction extend at 72°C for 5 minutes. 3µl of amplified products were electrophoresed on 0.8% agarose gel with ethidium bromide staining. M = 1 kb plus DNA size marker.

3.3.2 Obtaining the full-length sequence of Sj-PIG-N gene

The amplified full length of Sj-PIG-N PCR gene products were purified from agarose gel with QIAEX II Gel Extraction Kit as described in (referred to 2.2.3.4). 2μl of gene clean product was diluted with 18μl sterile dH_2O and then 4μl of diluted sample was used for cycle sequencing reaction. 1.28μl of 1.25pmol/μl of the sequencing primers were used in the reactions and followed the PCR profile described in (referred to 2.2.1.5). The locations and sequence information of sequencing primers were also showed in (Fig. 22). Combined the sequencing results from direct sequences on Sj-PIG-N gene, the full-length sequence of Sj-PIG-N gene was showed in (Fig. 22). The Sj-PIG-N gene has 3132bp including partial 5' and 3' untranslation region is. The coding region of Sj-PIG-N gene is 2964bp.

MF834→

agcgggaattcatcctggaggtcatgcatcaatttttagagttttgttcattgtcttactgtatttaatacaat base pairs
tcgcccttaagtaggacctccagtacgtagttaaaaatctcaaaaacaagtaacagaatgacataaattatgtta 1 to 75

MF867→

tttattccatattcgacatatattatacttcaccacttacacatggcgtaaacgttattcctttaaatatcagtg base pairs
aaataaggtataagctgtatataatatgaagtggtgaatgtgtaccgcatttgcaataaggaaatttatagtcac 76 to 150

ccccagcaacgcacatagttttaattgtttccgatggtcttcgcgcagacaaaatatttaatcatgagatggaat base pairs
ggggtcgttgcgtgtatcaaaattaacaaaggctaccagaagcgcgtctgttttataaattagtactctaccttа 151 to 225

ataccccttcctaagagatgtcctcttacaccgtggtgtatggggtgtgtcacatacgagagtccctaccgaat base pairs
tatggggaaaggattctctacaggagaatgtggcaccacataccccacacagtgtatgctctcagggatggctta 226 to 300

←MF868

caaggcctgcacatgtagctatactgggaggattctatgaagatgtagcctctataactaatgggtggaggacga base pairs
gttccggacgtgtacatcgatatgaccctcctaagatacttctacatcggagatattgattacccacctcctgct 301 to 375

atcctgtggagtttgataccgtactgaatcgttctaccctagcttggatatggggttacaaggaagttgtcatgt base pairs
taggacacctcaaactatggcatgacttagcaagatgggatcgaacctataccccaatgttccttcaacagtaca 376 to 450

MF806→

cctttgtaccacctttacacatcacatcaaagcaactccttgcccagatgaattaagtgatttggctaaaacta base pairs
ggaaacatggtggaaaatgtgtagtgtagtttcgttgaggaacgggtctacttaattcactaaaccgattttgat 451 to 525

atcctacagaaatcgaccgatgggtcaccgatcagtttacggacttcatcgataattcggatgacttctttgatg base pairs
taggatgtctttagctggctaccagtggctagtcaaatgcctgaagtagctattaagcctactgaagaaactac 526 to 600

←Mf809

accttactgcaatatctaatgattaccgtcagggtagaatggtatttctgcatttagatgctgctgatatggttg base pairs
tggaatgacgttatagattactaatggcagtcccatcttaccataaagacgtaaatctacgacgactataccaac 601 to 675

gtcattcttttaaacctgattcacctgaatatgttaaagttcttcgtaatttggacaaggtgattttccgtgttt base pairs
cagtaagaaaatttggactaagtggacttatacaatttcaagaagcattaaacctgttccactaaaaggcacaaa 676 to 750

atcagaagctaactgaaaaatctcgtggaagtgattcacgtatcgcttatatcctgacttctgatcatggaatga base pairs
tagtcttcgattgacttttagagcaccttcactaagtgcatagcgaatataggactgaagactagtaccttact 751 to 825

MF807→

ctgagtgggggaagtcatggatctggttctttgcatgaaacagtaacacctttgttagcttggggttctggtattg base pairs
gactcacccttcagtacctagaccaagaaacgtactttgtcattgtggaaacaatcgaaccccaagaccataac 826 to 900

←Mf808

tcggtccggtagaaatagaaactaatatgaataacttgtcgacagataaaaaagatgtgcttggtttaccactgc base pairs
agccaggccatctttatctttgattatacttattgaacagctgtctatttttctacacgaaccaaatggtgacg 901 to 975

ataattatggacgacttcgtcgtgaaattcagcaggcagatttatgccctttaatgtcgggtcttttaggcatcc base pairs
tattaatacctgctgaagcagcactttaagtcgtccgtctaaatacgggaaattacagcccagaaaatccgtagg 976 to 1050

caatcccagtcaattctataggtcaagttcctgttgaatttctaaagattcctgaatatgataaagtgaagttgg base pairs
gttagggtcagttaagatatccagttcaaggacaacttaaagatttctaaggacttatactatttcacttcaacc 1051 to 1125

MF869→

ttcgagcaaactggcttcaaatatatgcccaattaaagattaagtacacagaaaaaaagaaatctcatttcggta base pairs
aagctcgtttgaccgaagtttatatacgggttaatttctaattcatgtgtctttttttctttagagtaaagccat 1126 to 1200

ttttcttcagagaattcccatctttaaaaatgtcagatatttatgaaatggagaatatgtgtgaacttcttattt base pairs
aaaagaagtctcttaagggtagaaatttttacagtctataaatactttacctcttatacacacttgaagaataaa 1201 to 1275

ctagtggaaaatatcatgaggcaattcaagagtacagacatttaactagttcagcattgaaaggattgaactatt base pairs
gatcacctttttatagtactccgttaagttctcatgtctgtaaattgatcaagtcgtaactttcctaacttgataa 1276 to 1350

accataaatatgatcgattatacttagggttttgtgtttcgtcaacgttctgtttgtggagtctagtgatacttt base pairs
tggtatttatactagctaatatgaatcccaaaacacaaagcagttgcaagacaaacacctcagatcactatgaaa 1351 to 1425

gtcgtttattcattagaacagataatataaaccataaaaattatcaaagctatgcggatagtttctattggacag base pairs
cagcaaataagtaatcttgtctattatatttggtatttttaatagtttcgatacgcctatcaaagataacctgtc 1426 to 1500

←MF870

MF835→

tgattaatttcactgtgattggattcagtttattggttctttcttttgctaattcttggtctttgggacctacta base pairs
actaattaaagtgacactaacctaagtcaaataaccaagaaagaaaacgattaagaaccagaaaccctggatgat 1501 to 1575

tatatcagctagttccactcattttggtattatcattaagctatccaaaagctcgacgaaaacagttactaacat base pairs
atatagtcgatcaaggtgagtaaaaccataatagtaattcgataggttttcgagctgcttttgtcaatgattgta 1576 to 1650

tggtgggttacgtatggaaaggattattttctgattcgtcaacaacctcgtctttttttaccgtgtgttcaatca base pairs
accacccaatgcataccttcctaataaaagactaagcagttgttggagcagaaaaaaatggcacacaagttagt 1651 to 1725

catttatgtctatttgtatacttgagttacttgtttggggatttttccatcgttatctgctttctttgggttgtt base pairs
gtaaatacagataaacatatgaactcaatgaacaaacccctaaaaaggtagcaatagacgaaagaaacccaacaa 1726 to 1800

MF871→

tactactttcttcgtggccatatatagataagtcatttagaccacatgaaaagcacacacagatagttctttctt base pairs
atgatgaaagaagcaccggtatatatctattcagtaaatctggtgtacttttcgtgtgtgtctatcaagaaagaa 1801 to 1875

←MF872

tttggtgcgtttcatgttgttgtttggctatatttccttttattacctgtgattggatcaaatatgtatccaacga base pairs
aaaccacgcaaagtacaacaacaaaccgatataaaggaaataatggacactaacctagtttatacataggttgct 1876 to 1950

ttgtatttatgtctggtcttattcttacaccaattggaattataaccttacggtttgtatcaaattctcaaaatc base pairs
aacataaatacagaccagaataagaatgtggttaaccttaatattggaatgccaaacatagtttaagagttttag 1951 to 2025

atctttatatgttgcttggttatttttcggttttgttatttgtctttcaagttttgctgtttatgcaatgagtt base pairs
tagaaatatacaacgaaccaataaaaaagccaaaacaataaacagaaagttcaaaacgacaaatacgttactcaa 2026 to 2100

MF873→

ttccaatggtacgtactggatcattgaaaactattattcatatatacagttggtcagtgattatacttctaccct base pairs
aaggttaccatgcatgacctagtaacttttgataataagtatatatgtcaaccagtcactaatatgaagatggga 2101 to 2175

tgtcggtcattcttatcattccaacacagcttggacctcgagtggttggatggacagttgtttatcttgtcccac base pairs
acagccagtaagaatagtaaggttgtgtcgaacctggagctcaccaacctacctgtcaacaaatagaacagggtg 2176 to 2250

ttatactaatgagtactttctatgaagttacattctttgctgtattcgctgtagtcacctatctttggttatata base pairs
aatatgattactcatgaaagatacttcaatgtaagaaacgacataagcgacatcagtggatagaaaccaatatat 2251 to 2325

←MF874

tagaagttaaaaaacaaacgtctttgttaaagcatgataatatcaggttgtgggatattgaaacatcaactgaca base pairs
atcttcaatttttgtttgcagaaacaatttcgtactattatagtccaacaccctataactttgtagttgactgt 2326 to 2400

acacgtattcaaacattcagaatcaatttgccttctgtgaaaatttactaaccttgagaaattttcgtcagtcac base pairs
tgtgcataagtttgtaagtcttagttaaacggaagacacttttaaatgattggaactctttaaaagcagtcagtg 2401 to 2475

ttttctttatatttttctgacaatttcgtttttggtaccggaaatatcgctagtataaatagctttgatccac base pairs
aaaagaaatataaaaaagactgttaaagcaaaaaaccatggcctttatagcgatcatattttatcgaaactaggtg 2476 to 2550

←MF875

MF876→

gttcgacattctgtttcacaacaatattgaatcctgctttaatggcgattcttttattaataaaggtaatatgtc base pairs
caagctgtaagacaaagtgttgttataacttaggacgaaattaccgctaagaaaataattatttccattatacag 2551 to 2625

ctatgctatttttgggtgtaatctacgcagttattcagctgtgcaacgacagcttattcgactactcatcctttg base pairs
gatacgataaaaacccacattagatgcgtcaataagtcgacacgttgctgtcgaataagctgatgagtaggaaac 2626 to 2700

←MF836

tcaagtacataaacaatcgaaaatcaaaacgtggacatgattctattctagcacatactggtttaactgcagtac base pairs
agttcatgtatttgttagcttttagttttgcacctgtactaagataagatcgtgtatgaccaaattgacgtcatg 2701 to 2775

MF877→

tatctaatctaatagctatccacttttttctttggttgagagacgaaggtagttggttagatataggtactagta base pairs
atagattagattatcgataggtgaaaaaagaaaccaactctctgcttccatcaaccaatctatatccatgatcat 2776 to 2850

ttagtcattatgttatcgctatgtctataagtttggctgcatttctttttgcattattggggaaaaaaatgctga base pairs
aatcagtaatacaatagcgatacagatattcaaaccgacgtaaagaaaaacgtaataacccctttttttacgact 2851 to 2925
gcattcaaatgggtactaaaattcatcaagcacttttgaaagtatcgaacaagtatgtataaatacctttatgat base pairs
cgtaagtttacccatgattttaagtagttcgtgaaaactttcatagcttgttcatacatatttatggaaatacta 2926 to 3000

tatatcaagtaaatgatttttttaataagttaaatagctactttgtacctttacacttgtatatacaaagttattt base pairs
atatagttcatttactaaaaaattattcaatttatcgatgaaacatggaaatgtgaacatatatgtttcaataaa 3001 to 3075

←MF838

ctattcaagattaaagtaattttgttggaaaaaaaaaaaaaaaaaagaaaaaaaaaa base pairs
gataagttctaatttcattaaaacaacctttttttttttttttttctttttttttt 3076 to 3132

Fig. 22. Nucleotide sequence of Sj-PIG-N gene full length cDNA and locations of its gene specific primers. The full length sequence of Sj-PIG-N gene including 5' untranslation region and 3' untranslation region is 3132bp. The coding region of *S. japonicum* spans from 24-2987 nucleotides. The start codon "ATG" and stop codon "TAA" was boxed. The locations of the gene specific primers were also underlined on the sequence. Primers of MF838 and MF834 are the subcloning primers for full length Sj-PIG-N gene amplification. Primers of MF806, MF807, MF835, MF869, MF871, MF873, MF878, MF867 and MF877 are the sense sequencing primers. Primers of MF808, MF809, MF836, MF868, MF870, MF872, MF874 and MF875 are the anti-sense sequencing primers.

3.4 Sequence analysis of full length Sj-PIG-N cDNA

To further confirm the identity of the full-length cDNA, the nucleotide sequence was translated into amino acid sequence, which revealed that the coding region of Sj-PIG-N consisted of 987 amino acids (Fig. 23). And then, the translated amino acid sequence was compared with other protein sequences by Blastp. The result revealed that Sj-PIG-N protein might contain 6 putative conserved domains (Fig. 24). Except the conserved domain of AP superfamily of uncharacterized proteins (COG1524), the Sj-PIG-N protein also shares conserved domains of Phosphatidylinositolglycan class N (PIG-N) (Hong, *et al.*, 1999), Metalloenzyme - Metalloenzyme superfamily (Read, *et al.*, 2002), Phosphodiest- Type I phosphodiesterase / nucleotide pyrophosphatase (Kettenhofen, et al., 1998), GpmI, Phosphoglyceromutase (Valentin, 1993), and AslA, Arylsulfatase A and related enzymes (Gloeckner, *et al.*, 2003).

In addition, the amino acid sequence of Sj-PIG-N protein was compared with mouse phosphatidylinositolglycan class N (Hong, *et al.*, 1999), human phosphatidylinositolglycan class N (Strausberg *et al.*, 2002), *Saccharomyces cerevisiae* Mcd4p (Goffeau, et al., 1996) and *Caenorhabditis elegans* phosphatidylinositolglycan class N (Wilson, 1998) with CLUSTALW Multiple Sequence Alignment. Moreover, the three conserved motifs that Mcd4 proteins shared with phosphodiesterase and nucleotide pryophosphatases (Gaynor, *et al.*, 1999) were also found in Sj-PIG-N proteins in the alignment result (Fig 25). The alignment result revealed that the amino acid sequence of Sj-PIG-N and the four PIG-N proteins from different organisms shared about 30% identities and 50% similarities (Table 4).

Based on the amino acid sequence of Sj-PIG-N protein, a "KKML" amino acid

sequence was identified at the region 964-967 amino acid located at the C-terminal of Sj-PIG-N protein (Fig. 26). It is probably an ER retrieval motif sequence - "KKXX". The COOH-termianl double lysine motif maintains type I transmembrane proteins in the ER (Jackson *et al.*, 1993; Gaynor *e. al.*, 1994).

1 ATG CAT CAA TTT TTA GAG TTT TTG TTC ATT GTC TTA CTG TAT TTA 45

1 **Met** His Gln Phe Leu Glu Phe Leu Phe Ile Val Leu Leu Tyr Leu 15

46 ATA CAA TTT TAT TCC ATA TTC GAC ATA TAT TAT ACT TCA CCA CTT 90

16 Ile Gln Phe Tyr Ser Ile Phe Asp Ile Tyr Tyr Thr Ser Pro Leu 30

91 ACA CAT GGC GTA AAC GTT ATT CCT TTA AAT ATC AGT GCC CCA GCA 135

31 Thr His Gly Val Asn Val Ile Pro Leu Asn Ile Ser Ala Pro Ala 45

136 ACG CAC ATA GTT TTA ATT GTT TCC GAT GGT CTT CGC GCA GAC AAA 180

46 Thr His Ile Val Leu Ile Val Ser Asp Gly Leu Arg Ala Asp Lys 60

181 ATA TTT AAT CAT GAG ATG GAA TAT ACC CCT TTC CTA AGA GAT GTC 225

61 Ile Phe Asn His Glu **Met** Glu Tyr Thr Pro Phe Leu Arg Asp Val 75

226 CTC TTA CAC CGT GGT GTA TGG GGT GTG TCA CAT ACG AGA GTC CCT 270

76 Leu Leu His Arg Gly Val Trp Gly Val Ser His Thr Arg Val Pro 90

271 ACC GAA TCA AGG CCT GCA CAT GTA GCT ATA CTG GGA GGA TTC TAT 315

91 Thr Glu Ser Arg Pro Ala His Val Ala Ile Leu Gly Gly Phe Tyr 105

316 GAA GAT GTA GCC TCT ATA ACT AAT GGG TGG AGG ACG AAT CCT GTG 360

106 Glu Asp Val Ala Ser Ile Thr Asn Gly Trp Arg Thr Asn Pro Val 120

361 GAG TTT GAT ACC GTA CTG AAT CGT TCT ACC CTA GCT TGG ATA TGG 405

121 Glu Phe Asp Thr Val Leu Asn Arg Ser Thr Leu Ala Trp Ile Trp 135

406 GGT TAC AAG GAA GTT GTC ATG TCC TTT GTA CCA CCT TTT ACA CAT 450

136 Gly Tyr Lys Glu Val Val **Met** Ser Phe Val Pro Pro Phe Thr His 150

451 CAC ATC AAA GCA ACT CCT TGC CCA GAT GAA TTA AGT GAT TTG GCT 495

151 His Ile Lys Ala Thr Pro Cys Pro Asp Glu Leu Ser Asp Leu Ala 165

496 AAA ACT AAT CCT ACA GAA ATC GAC CGA TGG GTC ACC GAT CAG TTT 540

166 Lys Thr Asn Pro Thr Glu Ile Asp Arg Trp Val Thr Asp Gln Phe 180

541 ACG GAC TTC ATC GAT AAT TCG GAT GAC TTC TTT GAT GAC CTT ACT 585

181 Thr Asp Phe Ile Asp Asn Ser Asp Asp Phe Phe Asp Asp Leu Thr 195

586 GCA ATA TCT AAT GAT TAC CGT CAG GGT AGA ATG GTA TTT CTG CAT 630
196 Ala Ile Ser Asn Asp Tyr Arg Gln Gly Arg **Met** Val Phe Leu His 210

631 TTA GAT GCT GCT GAT ATG GTT GGT CAT TCT TTT AAA CCT GAT TCA 675
211 Leu Asp Ala Ala Asp **Met** Val Gly His Ser Phe Lys Pro Asp Ser 225

676 CCT GAA TAT GTT AAA GTT CTT CGT AAT TTG GAC AAG GTG ATT TTC 720
226 Pro Glu Tyr Val Lys Val Leu Arg Asn Leu Asp Lys Val Ile Phe 240

721 CGT GTT TAT CAG AAG CTA ACT GAA AAA TCT CGT GGA AGT GAT TCA 765
241 Arg Val Tyr Gln Lys Leu Thr Glu Lys Ser Arg Gly Ser Asp Ser 255

766 CGT ATC GCT TAT ATC CTG ACT TCT GAT CAT GGA ATG ACT GAG TGG 810
256 Arg Ile Ala Tyr Ile Leu Thr Ser Asp His Gly **Met** Thr Glu Trp 270

811 GGA AGT CAT GGA TCT GGT TCT TTG CAT GAA ACA GTA ACA CCT TTG 855
271 Gly Ser His Gly Ser Gly Ser Leu His Glu Thr Val Thr Pro Leu 285

856 TTA GCT TGG GGT TCT GGT ATT GTC GGT CCG GTA GAA ATA GAA ACT 900
286 Leu Ala Trp Gly Ser Gly Ile Val Gly Pro Val Glu Ile Glu Thr 300

901 AAT ATG AAT AAC TTG TCG ACA GAT AAA AAA GAT GTG CTT GGT TTA 945
301 Asn **Met** Asn Asn Leu Ser Thr Asp Lys Lys Asp Val Leu Gly Leu 315

946 CCA CTG CAT AAT TAT GGA CGA CTT CGT CGT GAA ATT CAG CAG GCA 990
316 Pro Leu His Asn Tyr Gly Arg Leu Arg Arg Glu Ile Gln Gln Ala 330

991 GAT TTA TGC CCT TTA ATG TCG GGT CTT TTA GGC ATC CCA ATC CCA 1035
331 Asp Leu Cys Pro Leu **Met** Ser Gly Leu Leu Gly Ile Pro Ile Pro 345

1036 GTC AAT TCT ATA GGT CAA GTT CCT GTT GAA TTT CTA AAG ATT CCT 1080
346 Val Asn Ser Ile Gly Gln Val Pro Val Glu Phe Leu Lys Ile Pro 360

1081 GAA TAT GAT AAA GTG AAG TTG GTT CGA GCA AAC TGG CTT CAA ATA 1125
361 Glu Tyr Asp Lys Val Lys Leu Val Arg Ala Asn Trp Leu Gln Ile 375

1126 TAT GCC CAA TTA AAG ATT AAG TAC ACA GAA AAA AAG AAA TCT CAT 1170
376 Tyr Ala Gln Leu Lys Ile Lys Tyr Thr Glu Lys Lys Lys Ser His 390

1171 TTC GGT ATT TTC TTC AGA GAA TTC CCA TCT TTA AAA ATG TCA GAT 1215
391 Phe Gly Ile Phe Phe Arg Glu Phe Pro Ser Leu Lys **Met** Ser Asp 405

1216 ATT TAT GAA ATG GAG AAT ATG TGT GAA CTT CTT ATT TCT AGT GGA 1260
406 Ile Tyr Glu **Met** Glu Asn **Met** Cys Glu Leu Leu Ile Ser Ser Gly 420

1261 AAA TAT CAT GAG GCA ATT CAA GAG TAC AGA CAT TTA ACT AGT TCA 1305
421 Lys Tyr His Glu Ala Ile Gln Glu Tyr Arg His Leu Thr Ser Ser 435

1306 GCA TTG AAA GGA TTG AAC TAT TAC CAT AAA TAT GAT CGA TTA TAC 1350
436 Ala Leu Lys Gly Leu Asn Tyr Tyr His Lys Tyr Asp Arg Leu Tyr 450

1351 TTA GGG TTT TGT GTT TCG TCA ACG TTC TGT TTG TGG AGT CTA GTG 1395
451 Leu Gly Phe Cys Val Ser Ser Thr Phe Cys Leu Trp Ser Leu Val 465

1396 ATA CTT TGT CGT TTA TTC ATT AGA ACA GAT AAT ATA AAC CAT AAA 1440
466 Ile Leu Cys Arg Leu Phe Ile Arg Thr Asp Asn Ile Asn His Lys 480

1441 AAT TAT CAA AGC TAT GCG GAT AGT TTC TAT TGG ACA GTG ATT AAT 1485
481 Asn Tyr Gln Ser Tyr Ala Asp Ser Phe Tyr Trp Thr Val Ile Asn 495

1486 TTC ACT GTG ATT GGA TTC AGT TTA TTG GTT CTT TCT TTT GCT AAT 1530
496 Phe Thr Val Ile Gly Phe Ser Leu Leu Val Leu Ser Phe Ala Asn 510

1531 TCT TGG TCT TTG GGA CCT ACT ATA TAT CAG CTA GTT CCA CTC ATT 1575
511 Ser Trp Ser Leu Gly Pro Thr Ile Tyr Gln Leu Val Pro Leu Ile 525

1576 TTG GTA TTA TCA TTA AGC TAT CCA AAA GCT CGA CGA AAA CAG TTA 1620
526 Leu Val Leu Ser Leu Ser Tyr Pro Lys Ala Arg Arg Lys Gln Leu 540

1621 CTA ACA TTG GTG GGT TAC GTA TGG AAA GGA TTA TTT TCT GAT TCG 1665
541 Leu Thr Leu Val Gly Tyr Val Trp Lys Gly Leu Phe Ser Asp Ser 555

1666 TCA ACA ACC TCG TCT TTT TTT ACC GTG TGT TCA ATC ACA TTT ATG 1710
556 Ser Thr Thr Ser Ser Phe Phe Thr Val Cys Ser Ile Thr Phe **Met** 570

1711 TCT ATT TGT ATA CTT GAG TTA CTT GTT TGG GGA TTT TTC CAT CGT 1755
571 Ser Ile Cys Ile Leu Glu Leu Leu Val Trp Gly Phe Phe His Arg 585

1756 TAT CTG CTT TCT TTG GGT TGT TTA CTA CTT TCT TCG TGG CCA TAT 1800
586 Tyr Leu Leu Ser Leu Gly Cys Leu Leu Leu Ser Ser Trp Pro Tyr 600

1801 ATA GAT AAG TCA TTT AGA CCA CAT GAA AAG CAC ACA CAG ATA GTT 1845
601 Ile Asp Lys Ser Phe Arg Pro His Glu Lys His Thr Gln Ile Val 615

1846 CTT TCT TTT TGG TGC GTT TCA TGT TGT TGT TTG GCT ATA TTT CCT 1890
616 Leu Ser Phe Trp Cys Val Ser Cys Cys Cys Leu Ala Ile Phe Pro 630

1891 TTA TTA CCT GTG ATT GGA TCA AAT ATG TAT CCA ACG ATT GTA TTT 1935
631 Leu Leu Pro Val Ile Gly Ser Asn **Met** Tyr Pro Thr Ile Val Phe 645

1936 ATG TCT GGT CTT ATT CTT ACA CCA ATT GGA ATT ATA ACC TTA CGG 1980
646 **Met** Ser Gly Leu Ile Leu Thr Pro Ile Gly Ile Ile Thr Leu Arg 660

1981 TTT GTA TCA AAT TCT CAA AAT CAT CTT TAT ATG TTG CTT GGT TAT 2025
661 Phe Val Ser Asn Ser Gln Asn His Leu Tyr **Met** Leu Leu Gly Tyr 675

2026 TTT TTC GGT TTT GTT ATT TGT CTT TCA AGT TTT GCT GTT TAT GCA 2070
676 Phe Phe Gly Phe Val Ile Cys Leu Ser Ser Phe Ala Val Tyr Ala 690

2071 ATG AGT TTT CCA ATG GTA CGT ACT GGA TCA TTG AAA ACT ATT ATT 2115
691 **Met** Ser Phe Pro **Met** Val Arg Thr Gly Ser Leu Lys Thr Ile Ile 705

2116 CAT ATA TAC AGT TGG TCA GTG ATT ATA CTT CTA CCC TTG TCG GTC 2160
706 His Ile Tyr Ser Trp Ser Val Ile Ile Leu Leu Pro Leu Ser Val 720

2161 ATT CTT ATC ATT CCA ACA CAG CTT GGA CCT CGA GTG GTT GGA TGG 2205
721 Ile Leu Ile Ile Pro Thr Gln Leu Gly Pro Arg Val Val Gly Trp 735

2206 ACA GTT GTT TAT CTT GTC CCA CTT ATA CTA ATG AGT ACT TTC TAT 2250
736 Thr Val Val Tyr Leu Val Pro Leu Ile Leu **Met** Ser Thr Phe Tyr 750

2251 GAA GTT ACA TTC TTT GCT GTA TTC GCT GTA GTC ACC TAT CTT TGG 2295
751 Glu Val Thr Phe Phe Ala Val Phe Ala Val Val Thr Tyr Leu Trp 765

2296 TTA TAT ATA GAA GTT AAA AAA CAA ACG TCT TTG TTA AAG CAT GAT 2340
766 Leu Tyr Ile Glu Val Lys Lys Gln Thr Ser Leu Leu Lys His Asp 780

2341 AAT ATC AGG TTG TGG GAT ATT GAA ACA TCA ACT GAC AAC ACG TAT 2385
781 Asn Ile Arg Leu Trp Asp Ile Glu Thr Ser Thr Asp Asn Thr Tyr 795

2386 TCA AAC ATT CAG AAT CAA TTT GCC TTC TGT GAA AAT TTA CTA ACC 2430
796 Ser Asn Ile Gln Asn Gln Phe Ala Phe Cys Glu Asn Leu Leu Thr 810

2431 TTG AGA AAT TTT CGT CAG TCA CTT TTC TTT ATA TTT TTT CTG ACA 2475
811 Leu Arg Asn Phe Arg Gln Ser Leu Phe Phe Ile Phe Phe Leu Thr 825

2476 ATT TCG TTT TTT GGT ACC GGA AAT ATC GCT AGT ATA AAT AGC TTT 2520
826 Ile Ser Phe Phe Gly Thr Gly Asn Ile Ala Ser Ile Asn Ser Phe 840

2521 GAT CCA CGT TCG ACA TTC TGT TTC ACA ACA ATA TTG AAT CCT GCT 2565
841 Asp Pro Arg Ser Thr Phe Cys Phe Thr Thr Ile Leu Asn Pro Ala 855

2566 TTA ATG GCG ATT CTT TTA TTA ATA AAG GTA ATA TGT CCT ATG CTA 2610
856 Leu **Met** Ala Ile Leu Leu Leu Ile Lys Val Ile Cys Pro **Met** Leu 870

2611 TTT TTG GGT GTA ATC TAC GCA GTT ATT CAG CTG TGC AAC GAC AGC 2655
871 Phe Leu Gly Val Ile Tyr Ala Val Ile Gln Leu Cys Asn Asp Ser 885

2656 TTA TTC GAC TAC TCA TCC TTT GTC AAG TAC ATA AAC AAT CGA AAA 2700
886 Leu Phe Asp Tyr Ser Ser Phe Val Lys Tyr Ile Asn Asn Arg Lys 900

2701 TCA AAA CGT GGA CAT GAT TCT ATT CTA GCA CAT ACT GGT TTA ACT 2745
901 Ser Lys Arg Gly His Asp Ser Ile Leu Ala His Thr Gly Leu Thr 915

2746 GCA GTA CTA TCT AAT CTA ATA GCT ATC CAC TTT TTT CTT TGG TTG 2790
916 Ala Val Leu Ser Asn Leu Ile Ala Ile His Phe Phe Leu Trp Leu 930

2791 AGA GAC GAA GGT AGT TGG TTA GAT ATA GGT ACT AGT ATT AGT CAT 2835
931 Arg Asp Glu Gly Ser Trp Leu Asp Ile Gly Thr Ser Ile Ser His 945

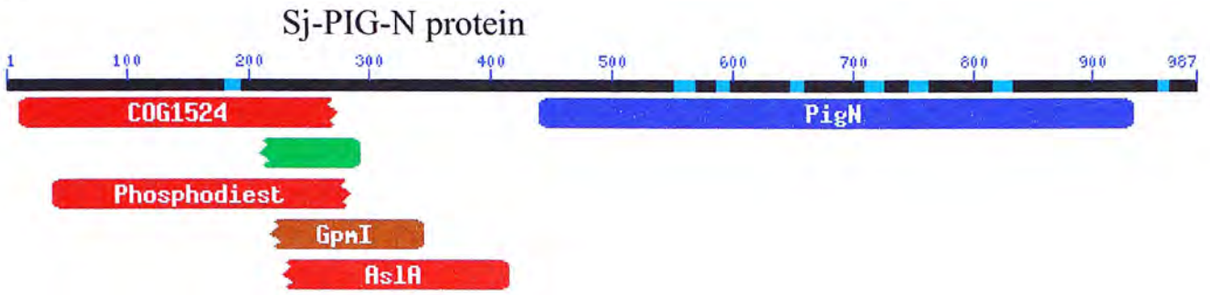
2836 TAT GTT ATC GCT ATG TCT ATA AGT TTG GCT GCA TTT CTT TTT GCA 2880
946 Tyr Val Ile Ala **Met** Ser Ile Ser Leu Ala Ala Phe Leu Phe Ala 960

2881 TTA TTG GGG AAA AAA ATG CTG AGC ATT CAA ATG GGT ACT AAA ATT 2925
961 Leu Leu Gly Lys Lys **Met** Leu Ser Ile Gln **Met** Gly Thr Lys Ile 975

```
2926  CAT CAA GCA CTT TTG AAA GTA TCG AAC AAG TAT GTA TAA   2964
976   His Gln Ala Leu Leu Lys Val Ser Asn Lys Tyr Val End   987
```

Fig. 23. Translation of full length Sj-PIG-N nucleotide sequence to amino acid sequence. The coding region of Sj-PIG-N protein contains 2964 nucleotides, which consists of 987 amino acids.

(a)



(b)

		Score (bits)	E value
gnl|CDD|11238	COG1524, COG1524, Uncharacterized proteins of the AP superfami...	87.6	6e-18
gnl|CDD|17565	pfam04987, PigN, Phosphatidylinositolglycan class N (PIG-N). P...	75.4	3e-14
gnl|CDD|17020	pfam01676, Metalloenzyme, Metalloenzyme superfamily. This fami...	46.0	2e-05
gnl|CDD|17018	pfam01663, Phosphodiest, Type I phosphodiesterase / nucleotide...	43.4	1e-04
gnl|CDD|10565	COG0696, GpmI, Phosphoglyceromutase [Carbohydrate transport an...	42.5	3e-04
gnl|CDD|12458	COG3119, AslA, Arylsulfatase A and related enzymes [Inorganic ...	38.2	0.005

Fig. 24. BLASTP search result of Sj-PIG-N full length protein. Graph (a) showed the relative positions of conserved domains on Sj-PIG-N protein. Table (b) showed the detail information of the six conserved domains. Except conserved domain of AP superfamily of uncharacterized proteins (COG1524), the Sj-PIG-N protein also shares conserved domains of Phosphatidylinositolglycan class N (PIG-N), Metalloenzyme - Metalloenzyme superfamily, Phosphodiest- Type I phosphodiesterase / nucleotide pyrophosphatase, GpmI, Phosphoglyceromutase, and AslA, Arylsulfatase A and related enzymes

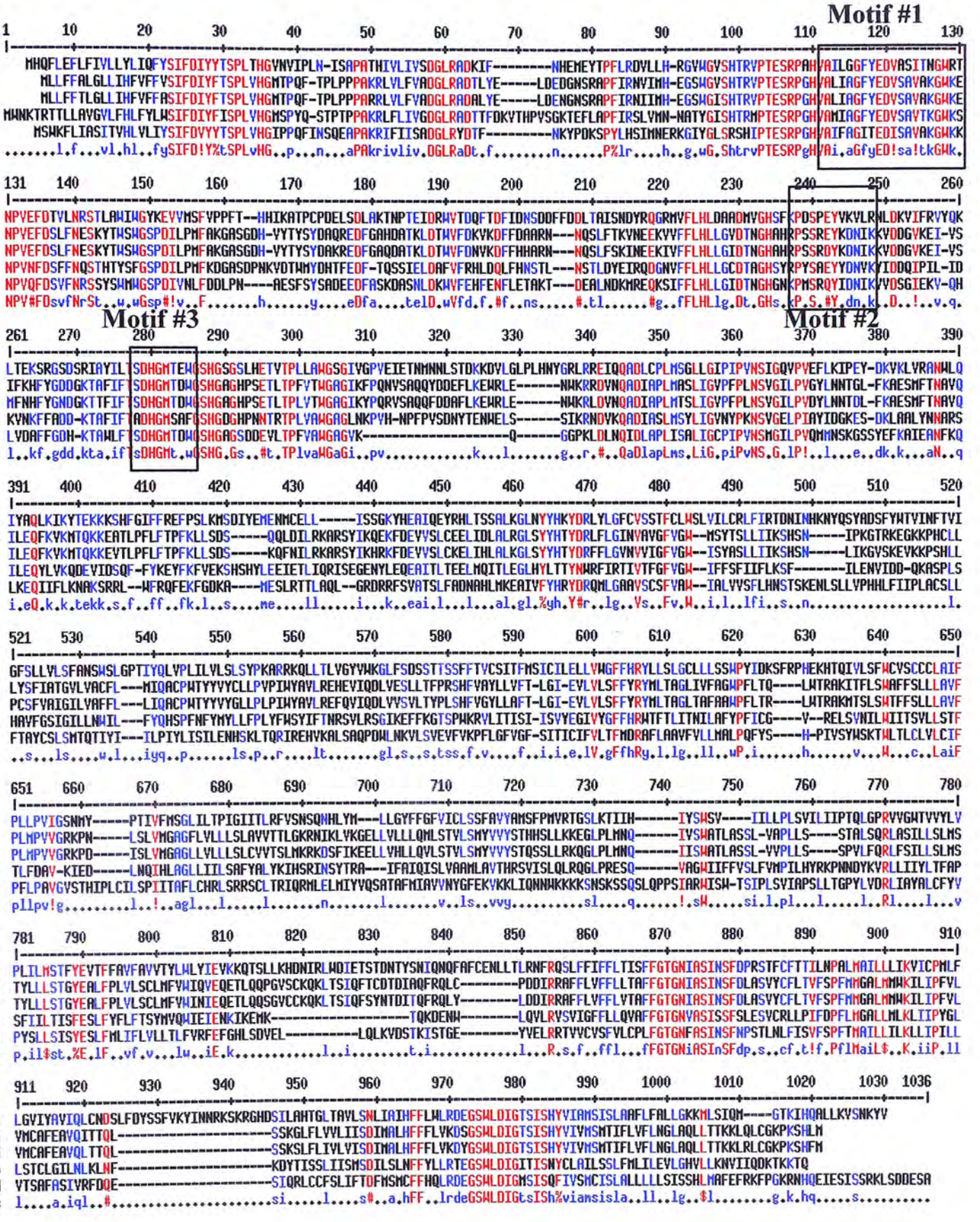


Fig. 25. Multiple alignment of the amino acid sequences of Sj-PIG-N, mouse PIG-N, human PIG-N, yeast MCD4p, and *C. elegan* PIG-N. The Three boxes are the shared conserved motifs of Sj-PIG-N and other PIG-N proteins.

Table 4. The homology of amino acid sequence of of Sj-PIG-Nm, mouse PIG-N, human PIG-N, MCD4p, and *C. elegan* PIG-N.

	Sj-PIG-N	
	Identity	Similarity
Mouse PIG-N	28%	46%
Human PIG-N	29%	46%
Yeast MCD4	31%	48%
Caenorhabditis elegans PIG-N	33%	52%

Fig. 26. The location of amino acids 'KKXX' ER retrieval motif in Sj-PIG-N protein. The ER retrieval motif sequence of Sj-PIG-N " KKML " is located at amino acid 964 - 967.

3.5 Analysis of Sj-PIG-N protein structure by computer programs

For the study of Sj-PIG-N gene, the protein sequence was analyzed with computer programs. The PIG-N is an enzyme that transfers an EtN-P to the first mannose of GPI anchor main core. It was already proved that the location of PIG-N protein is on the ER membrane (Gaynor *et. al.,* 1999). The computer program SignalP V2.0 (Nielsen, *et al.*, 1997) was applied to predict the signal peptide cleavage sites of Sj-PIG-N protein. This prediction indicated that the most likely cleavage site of signal peptide should be between amino acid 21 and 22: YSI-FD (Fig. 27). That means the length of putative signal peptide of Sj-PIG-N protein should be 21 amino acids (Fig. 28).

Being PIG-N homologous gene, it is reasonable to believe that Sj-PIG-N gene is a membrane protein. For the detection of trans-membrane domain, a transmembrane domain prediction program TopPred2 (Von Heijne, 1992; Claros and Von Heijne, 1994) was used to predict the topology of Sj-PIG-N. There were sixteen hydrophobic domains identified by the program (Fig. 29). In addition, for the N-linked glycosylation sites prediction, the amino acid sequence of Sj-PIG-N was applied in N-linked glycosylation sites prediction program NetNGlyc 1.0 (Gupta, *et al.*, 2002). There were four N-linked glycosylation sites detected in Sj-PIG-N protein (Fig. 30).

Combined the predications of TopPred2 and NetNGlyc1.0, the predicted topology graph of Sj-PIG-N protein was drawn (Fig. 31). There are sixteen predicted trans-membrane domains (TMDs) in Sj-PIG-N. Two of the four N-linked glycosylation sites located at the first large N-terminal hydrophilic region; the third one followed the second predicted TMD, and last one preceded the fifth TMD.

Moreover, the C-terminus of the protein contains the amino acids 'KKXX' ER retrieval motif that located within the last TMD.

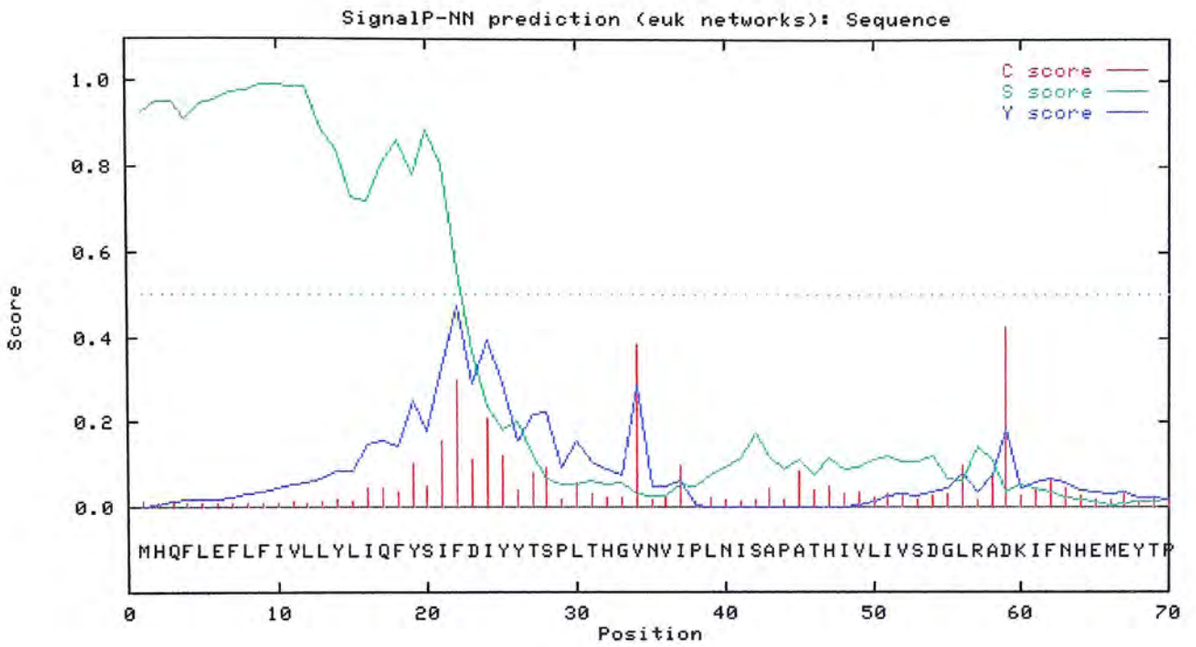


Fig. 27. Predicted signal peptide cleavage sites of Sj-PIG-N protein. The cleavage site of signal peptide should be between amino acid 21 and 22: YSI-FD. The maximum value of raw cleavage site score (C-score) is 0.424, the maximum value of signal peptide (S-score) score is 0.993, and the mean value of signal peptide score (mean S-score) is 0.900 and the geometric average between the C-score and a smoothed derivative of the S-score (Y-score). (Http://www.cbs.dtu.dk/services/signalp; Nielsen, *et al.*, 1997).

"M H Q F L E F L F I V L L Y L I Q F Y S I"

Fig. 28. The amino acid sequence of predicted signal peptide. The length of signal peptide is 21 amino acids, and the position of signal peptide is from position 1 to position 21 in Sj-PIG-N protein.

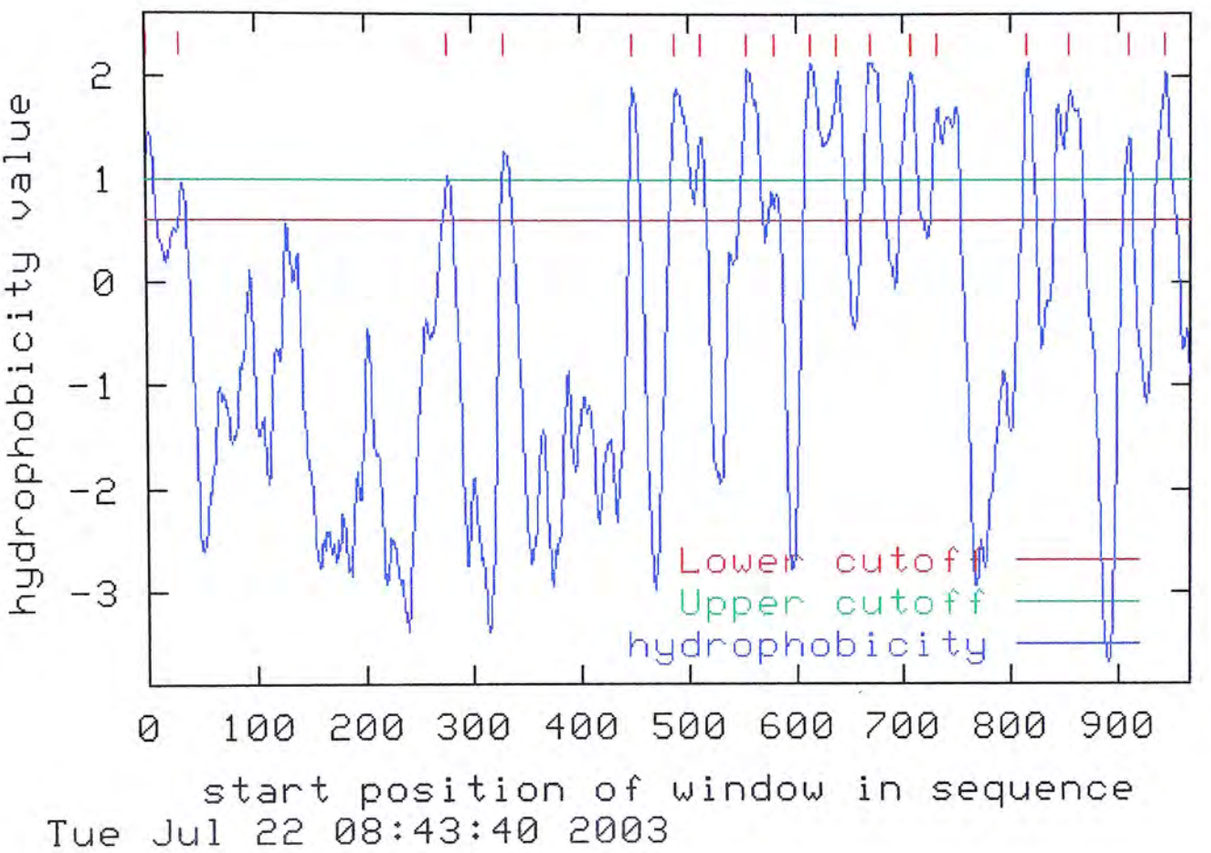


Fig. 29. Hydropathy profile of Sj-PIG-N drawn by the membrane proteins topology prediction program - TopPred2. Above the upper cutoff line, there are sixteen hydrophobic regions within Sj-PIG-N protein. (Http://www.sbc.cu.se/~erikw/toppred2; Von Heijne, 1992; Claros and Von Heijne, 1994)

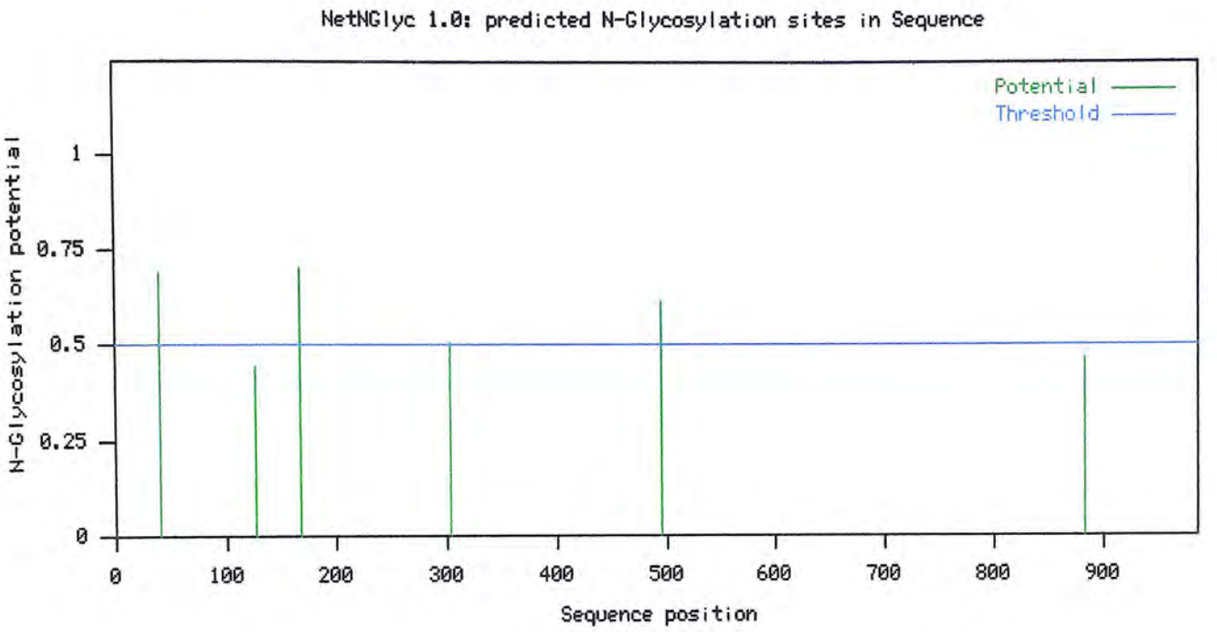


Fig. 30. Predicted potential N-linked glycosylation sites of Sj-PIG-N, based on the prediction of NetNGlyc 1.0. The Four predicted N-glycosylation sites: "NIS" (amino acid 42 - 44), "NPT" (amino acid 168 - 170), NLS (amino acid 304 - 306), and NFT (amino acid 495 - 497). (Http://www.cbs.dtu.dk/services/netnglyc; Gupta, *et al.*, 2002)

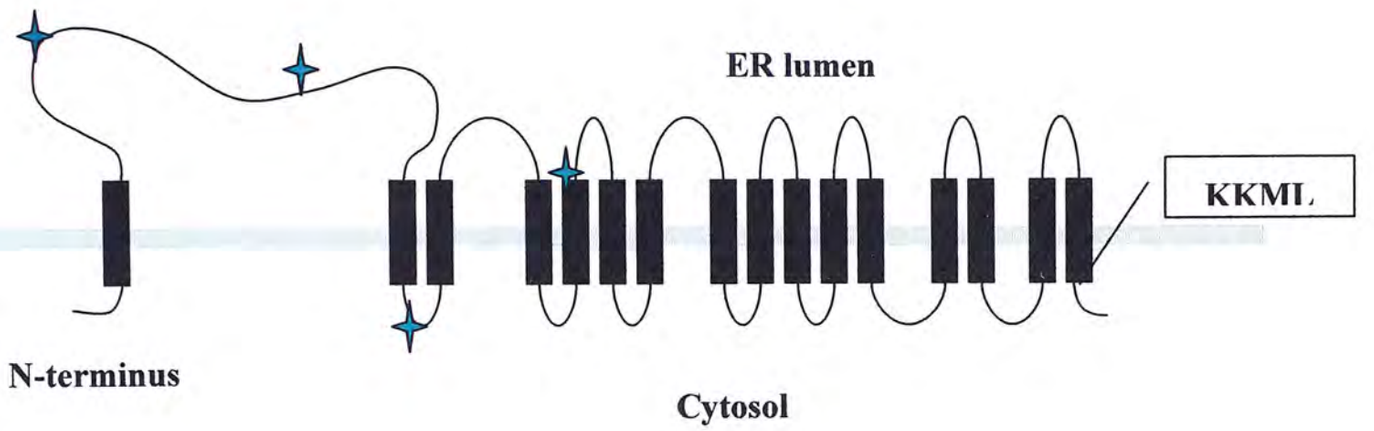


Fig. 31. Predicted topology of Sj-PIG-N in the ER membrane, based on the prediction of TopPred2 program and NetNGlyc 1.0. Transmembrane Domains – TMDs (black boxex), N-linked glycosylation sites likely to be utilized (✦) , and the C-terminal "KKXX" motif was shown. There are sixteen predicted TMDs in Sj-PIG-N. The 4 N-linked glycosylation sites: two within the large N-terminal hydrophilic region, one following the second predicted TMD, and one preceding the fifth TMD. The C termus of the protein contains the amino acids 'KKML' ER retrieval motif that is found within the last TMD.

3.6 Construction of Sj-PIG-N gene into mammalian expression vector

3.6.1 Construction of pBluescriptII KS(+)-Sj-PIG-N-B

For the cloning of Sj-PIG-N gene, the first step was to clone sj-PIG-N into pBluescriptII to construct the pBluescriptII KS(+)-Sj-PIG-N-B and then sub-cloned into other designed expression vectors. Primers MF933 and MF935 were designed to clone Sj-PIG-N-B gene. Within the primer MF933, a linker sequence containing NcoI was linked to the 5' end of the N-terminal primer of Sj-PIG-N. There was a linker sequence of BamHI attached at the 5'end of C-terminal primer – MF935. The full length gene was firstly sub-cloned into pBluescriptII KS(+) vector (Fig. 32). The target gene was successfully amplified by RT-PCR with specific primers MF 933 and MF 935 (Fig. 33). The PCR product was purified from agarose gel and ligated into pBluescriptII KS(+) T vector (referred to 2.2.3.4 and 2.2.3.5). After that, the full length sequences of the clones of pBluescriptII KS(+)-Sj-PIG-N-B were sequenced as described in (2.2.1.5). However, in the Polymerase Chain Reaction, Taq DNA polymerase creates one incorrect base in every 10,000 base pairs that leads to a high mutation rate. For each of the pBluescriptII KS(+)-Sj-PIG-N-B clones, it contains 4 to 7 incorrect bases. To obtain a clone carrying a full length gene of Sj-PIG-N without mutation, 7 fragments without sense mutation of Sj-PIG-N gene from different pBluescriptII KS(+)-Sj-PIG-N–B clones were sub-cloned into a new clone carrying Sj-PIG-N gene (Fig. 34).

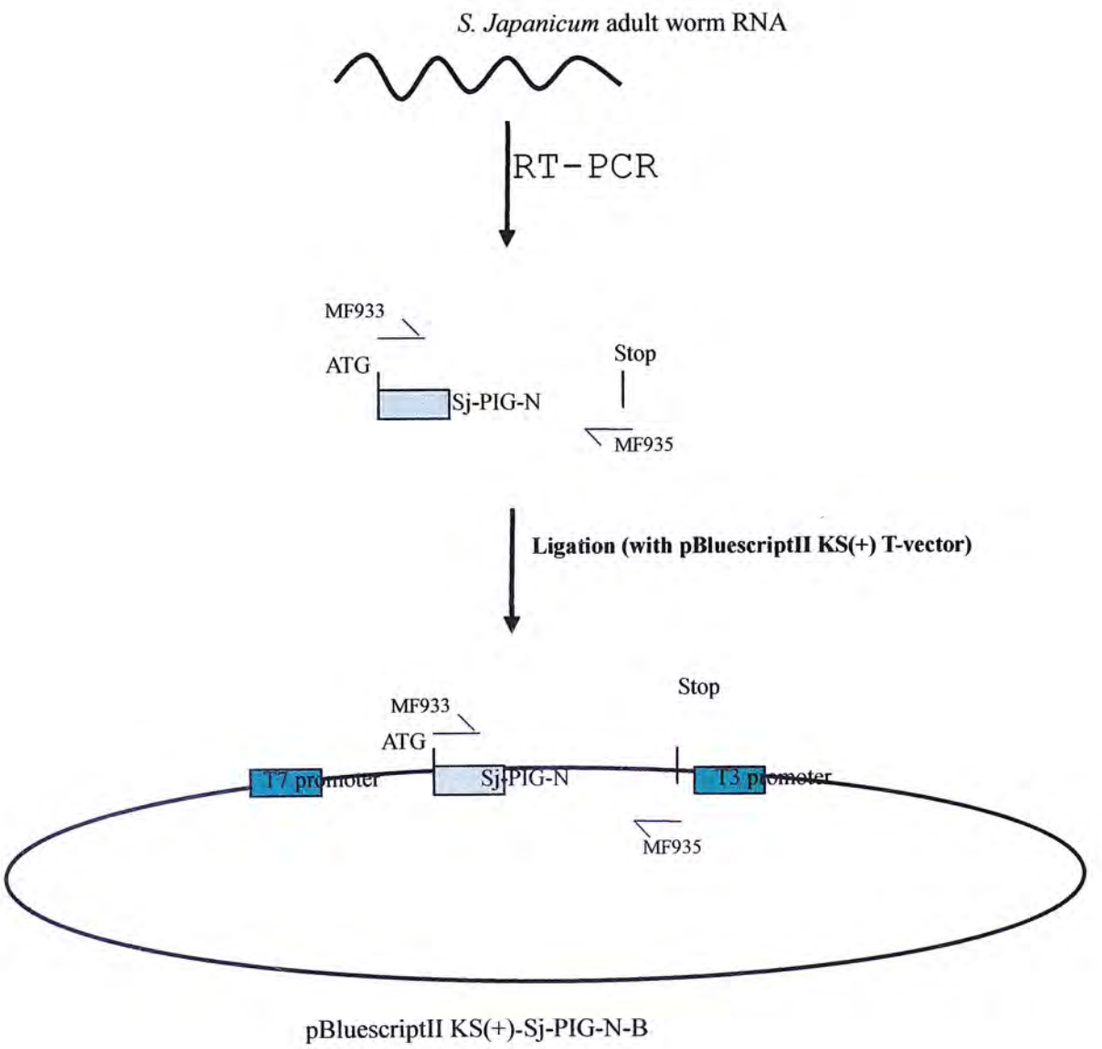


Fig. 32. Outline of construction of pBluescriptII KS(+)-Sj-PIG-N-B.

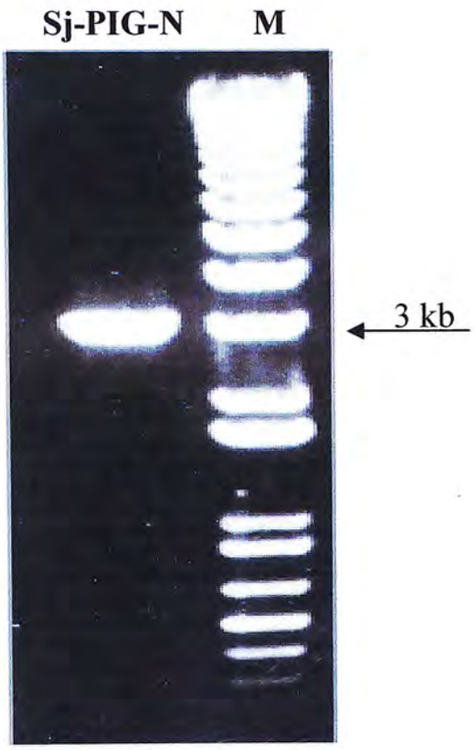


Fig. 33. Amplification of Sj-PIG-N full-length gene with gene specific primers of MF933 and MF935. The resulting PCR product was about 3.0kb. The total volume of reaction mix is 15µl. The template DNA was denatured at 94°C for 3 minutes and then amplified for 35 cycles with a cycle profile: 94°C for 30 seconds, 61°C for 30 seconds and 72°C for 3.5 minutes. After the last cycle, the reaction extend at 72°C for 5 minutes. 3µl of amplified products were electrophoresed on 0.8% agarose gel with 0.25 µg / ml ethidium bromide. M = 1 kb plus DNA size marker.

pBluescriptII KS(+) cloning region

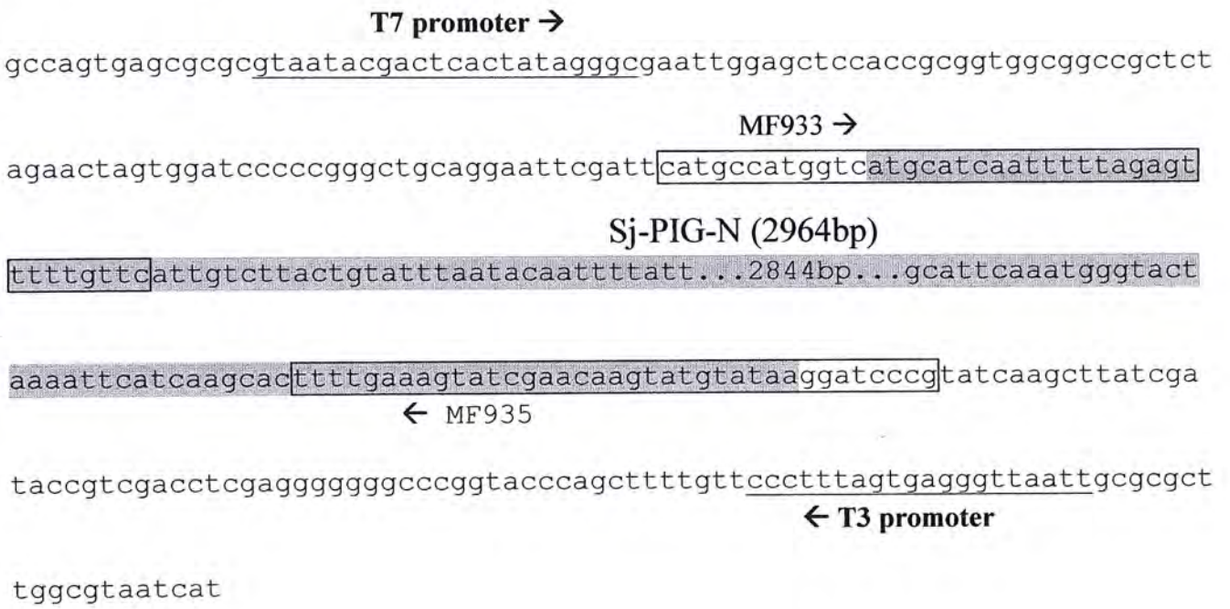


Fig. 34. Nucleotide sequences of the cloning region of pBluescript II KS(+)-Sj-PIG-N-B clone. The shadow area is the coding region of Sj-PIG-N gene. The sequence of T7 and T3 promoter were underlined.

3.6.2 Construction of pBluescriptII KS(+)-Sj-PIG-N-E

For the mammalian cell expression of Sj-PIG-N protein, the first step was to clone sj-PIG-N into pBluescriptII KS(+) to construct the pBluescriptII KS(+)-Sj-PIG-N-E (Fig. 35) and then sub-cloned into other designed expression vectors. In order to express the Sj-PIG-N gene in mammalian cell, primers MF934 was designed to clone the gene into mammalian cell expression vector. A kozak consensus sequence (Kozak, 1987) were linked to the 5' end of N-terminal primer MF934 to increase the translation efficiency of Sj-PIG-N gene in eukaryotic cell.

In the construction of pBluescriptII KS(+)-Sj-PIG-N-E, the N-terminal of Sj-PIG-N gene was amplified with RT-PCR using primer – MF934 and 805 (Fig. 36). The N-terminal fragment was cloned into pBluescriptII KS(+) T vector. After confirmed the fragment sequence within pBluescriptII KS(+)/Sj-PIG-N/MF934/805, it was excised with XbaI and BstEII then sub-cloned into a XbaI and BstEII cut pBluescriptII KS(+)-Sj-PIG-N-B vector.

10μg of pBlusecriptII KS(+)/Sj-PIG-N/MF935-MF805 was digested with 20 units of XbaI in 80μl reaction mix of 1x buffer D and incubated at 37°C for 3 hours. After purified by ethanol precipitation, it was dissovled in 40μl Milli-Q water. Then, 40μl of XbaI cut products was digested with 20 units of BstEII in 70μl final volume of 1x buffer D at 60°C for 3 hours. The double enzymes digested product was purifie for agarose gel (referred to 2.2.3.4) and finally resuspended in 20 Milli-Q water. The vector, pBluescriptII KS(+)-Sj-PIG-N-B was prepared by digested with XbaI and BstEII. After enzyme digestion, then XbaI and BstEII pBluescriptII

KS(+)-Sj-PIG-N-B was purified by Qiagen gel extraction kit from agarose gel (referred to 2.2.3.4) and resuspended in 20μl Milli-Q water. 6μl of XbaI and BstEII cut Sj-PIG-N fragment was ligated with 1μl of XbaI and BstEII pBluescriptII KS(+) △Sj-PIG-N-B in a final volume of 10μl reaction mix. The ligation product was transformed into DH5α competent cells by heat shock transformation. The sequence of cloning region of pBluescriptII KS(+)-Sj-PIG-N-E was showed in (Fig. 37).

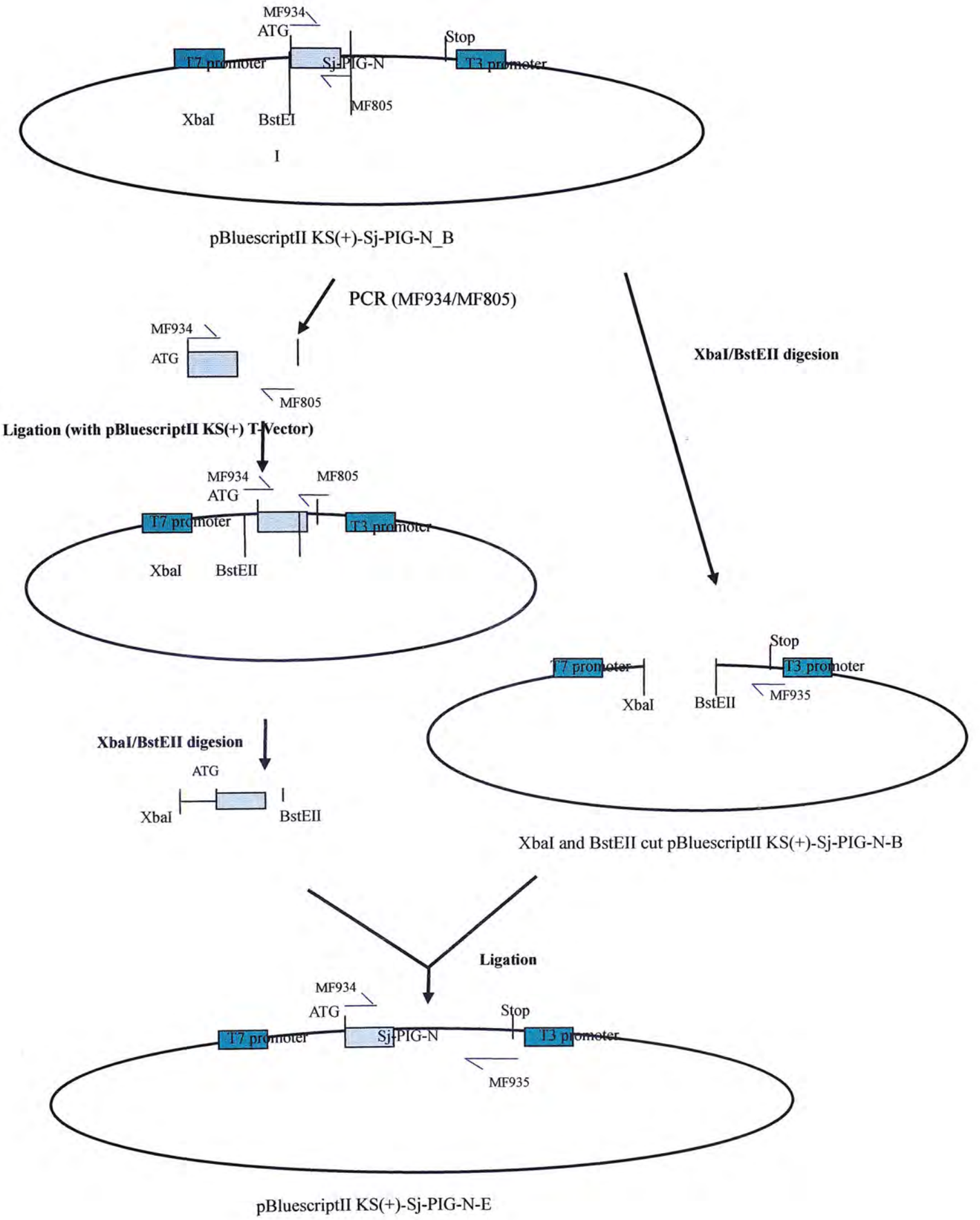

MF934
ATG
Stop
T7 promoter
Sj-PIG-N
T3 promoter
MF805
XbaI
BstEI
I
pBluescriptII KS(+)-Sj-PIG-N_B
PCR (MF934/MF805)
XbaI/BstEII digesion
MF934
ATG
MF805
Ligation (with pBluescriptII KS(+) T-Vector)
MF934
MF805
ATG
T7 promoter
T3 promoter
XbaI
BstEII
Stop
T7 promoter
T3 promoter
XbaI
BstEII
MF935
XbaI/BstEII digesion
ATG
XbaI
BstEII
XbaI and BstEII cut pBluescriptII KS(+)-Sj-PIG-N-B
Ligation
MF934
ATG
Stop
T7 promoter
Sj-PIG-N
T3 promoter
MF935
pBluescriptII KS(+)-Sj-PIG-N-E

Fig. 35. Outline of construction of pBluescriptII KS(+)-Sj-PIG-N-E.

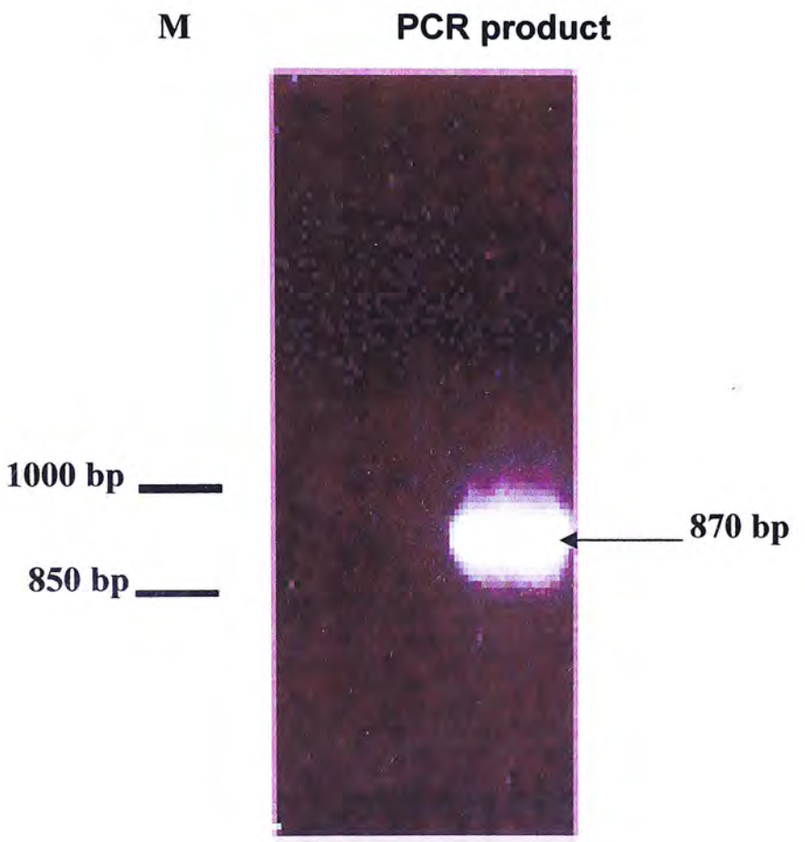


Fig. 36. N-terminal of Sj-PIG-N gene was amplified by RT-PCR with MF934 and MF805. The total volume of PCR reaction was 15µl. The template DNA was denatured at 94˚C for 3 minutes and then amplified for 25 cycles with a cycle profile: 94˚C for 30 seconds, 64˚C for 30 seconds and 72˚C for 1 minutes. After the last cycle, the reaction extend at 72˚C for 3 minutes. 5µl of amplified products were electrophoresed on 1% agarose gel with ethidium bromide staining. M = 1 kb plus DNA size marker.

pBluescriptII KS(+) cloning region

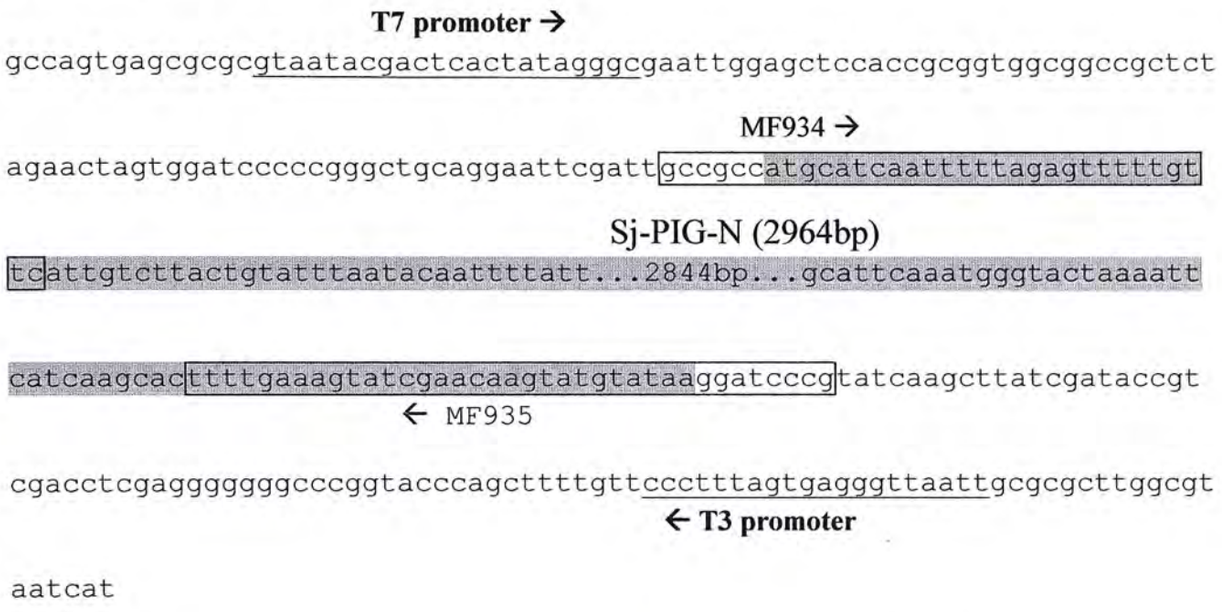


Fig. 37. Nucleotide sequences of the cloning region of pBluescriptII KS(+)-Sj-PIG-N-E. The shadow area is the coding region of Sj-PIG-N gene. The location of T7 and T3 promoter were underlined.

3.6.3 Construction of pTRE2-Sj-PIG-N

Sj-PIG-N gene was firstly sub-cloned into pTRE2 vector from pBluescriptII KS(+)-Sj-PIG-N-E and then cloned into mammalian cell expression vector. To construct the pTRE2-Sj-PIG-N, Sj-PIG-N was excised with BamHI and HindIII and cloned into pTRE2 vector (Fig. 38).

Sj-PIG-N was cut from pBluescriptII KS(+)-Sj-PIG-N-E by restriction enzyme BamHI and HindIII. 6μg of plasmid was digested with 15 units BamHI in 40μl final volume of 1x one for all buffer and incubated at 37°C for 3 hours. The digested plasmid were purified by Qiagen gel extraction kit from agarose gel (referred to 2.2.3.4) and resuspended in 15μl Milli-Q water. 10μl of BamHI cut plasmid was digested with 15 units of HindIII in 40μl reaction mix of 1x buffer E. The reaction was incubated at 37°C for 3 hours. BamHI and HindIII digested Sj-PIG-N was purified Qiagen gel extraction kit from agarose gel (referred to 2.2.3.4) and resuspended in 10μl Milli-Q water.

Restriction enzymes – BamHI and HindIII were also used in the preparation of vector, pTRE2. 1μg of pTRE2 was digested with 5 units BamHI in 20μl final volume of 1x one for all buffer and incubated at 37°C for 3 hours. 10μl purified of BamHI cut pTRE2 was digested with 5 units of HindIII in 20μl reaction mix of 1x buffer E. The reaction was incubated at 37°C for 3 hours. 6μl of purified Sj-PIG-N gene was ligated with 1μl of purified BamHI and HindIII digested pTRE2 in a final volume of 10μl reaction mix. The ligation product was transformed into DH5α competent cells by

heat shock transformation (referred to 2.2.3.7). After confirmed its sequence, the construct, pTRE2-Sj-PIG-N, was ready for further sub-cloning (Fig. 39).

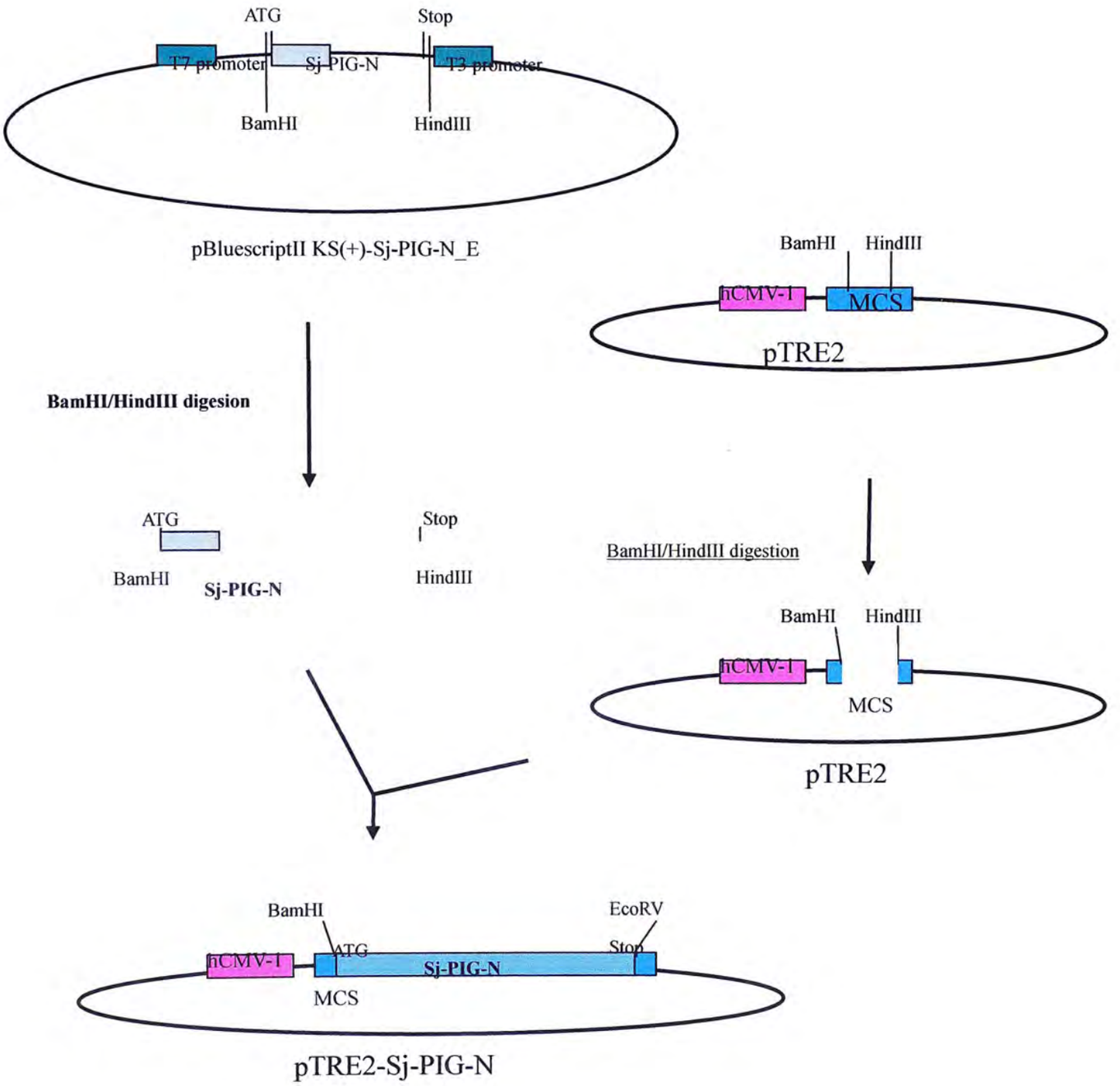


Fig. 38. Outline of construction of pTRE2-Sj-PIG-N.

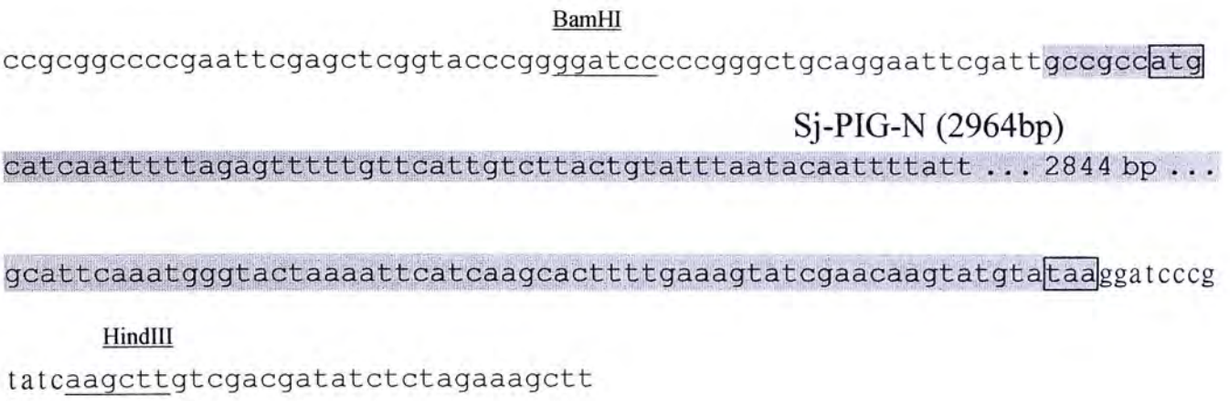


Fig. 39. Nucleotide sequences of cloning region of pTRE2-Sj-PIG-N. The Sj-PIG-N gene was cloned into pTRE2 vector by BamHI and HindIII digestion.

3.6.4 Construction of pEGFP-Hyg-Sj-PIG-N

3.6.4.1 Construction of pEGFP-Hyg

Mammalian cell expression vector was constructed with part of pEGFP-N1 carrying multiple cloning sites region and the part of pTK-Hyg containing hygromycine resistant gene. pEGFP-N1 was digested with AflII and AflIII, and the 5' protruding ends were filled in by T4 DNA polymerase to generate blunt ends. In the Fill-in reaction, 20μl digested sample was fill-in by 6 units of T4 DNA polymerase in 50μl final volume of 1x DNA polymerase buffer with the addition of 2μl of 10mM dNTPs, 50μg/ml BSA. The reaction mix was incubated at 20°C for 20 minutes to maximize the ratio of polymerase activity to the exonuclease activity. Heat-inactivation was then performed by incubationg at 65°C for 15 minutes. Finally, digested pEGFP-N1 was ligated with SspI digested pTK-Hyg to construct the pEGFP-Hyg construct (Fig. 40).

Digestion mapping was used to identify positive transformants and the orientation of enhanced green fluorescent protein in pEGFP-Hyg with HindIII. As only one restriction site of HindIII is located in both the multiple cloning site of pEGFP-N1 sequence and pTK-Hyg fragment, there were 2 HindIII restriction sites located on pEGFP-Hyg vector. Moreover, the fragments size of HindIII digested pEGFP-Hyg can also prove the orientation of enhanced green fluorescent protein in the vector. Detail information was showed in (table 5). Based on the endonuclease restriction result (Fig. 41), the pEGFP-Hyg construct belonged to the construct type

(a). In the construct type (a), the C-terminal of enhanced green florescent protein faced to the C-terminal of hygromycine resistance protein.

In order to test whether the pEGFP-Hyg can express in mammalian cells or not, the plasmid DNA of pEGFP-Hyg were transient transfected into a mouse cell line – 3T12 by DMRIE-C reagent as described in (referred to 2.2.4.2). About $2x10^6$ 3T12 cells were seeded in a 35-mm tissue culture dish. When the cell density reached 60% confluent, a mixture containing 2μg of pEGFP-Hyg and 4μl of DMIRE-C reagent was transferred into the culture dish and incubated for 5 hours to allow the DNA enter the cells. After 24 hours of the transient transfection, the image was captured under fluorescent microscope with 505-550-nm filter (Fig. 42). The result indicated that the enhanced green fluorescent protein within the pEGFP-Hyg vector could be expressed within mammalian cells.

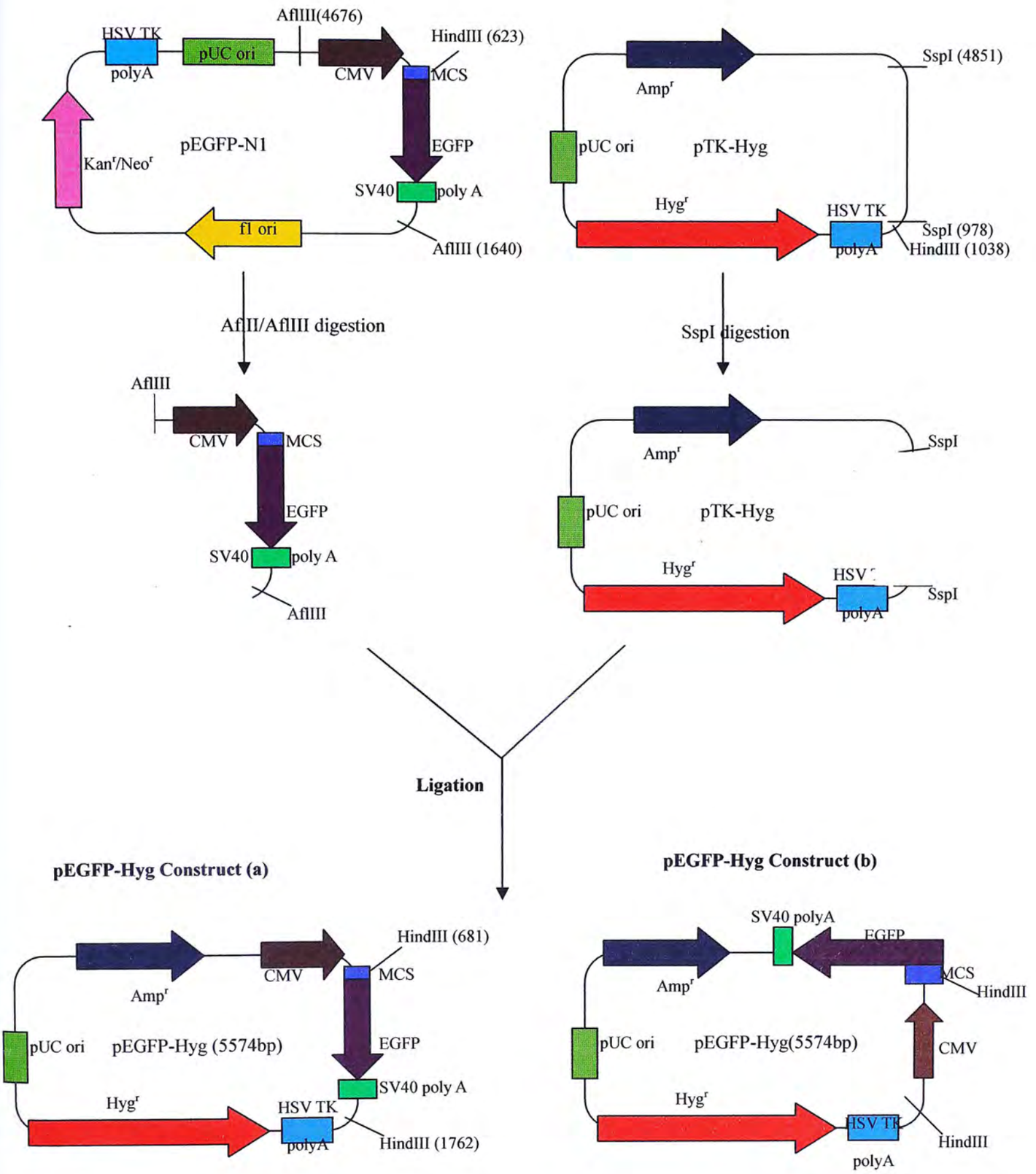


Fig. 40. Outline of construction of pEGFP-Hyg.

Table 5. Predicted HindIII restriction results of pEGFP-Hyg construct (a) and pEGFP-Hyg construct (b).

Restriction Enzyme	pEGFP-Hyg Construct (a)	pEGFP-Hyg Construct (b)
HindIII	4.4 kb 1.1 kb	0.7 kb 4.8 kb

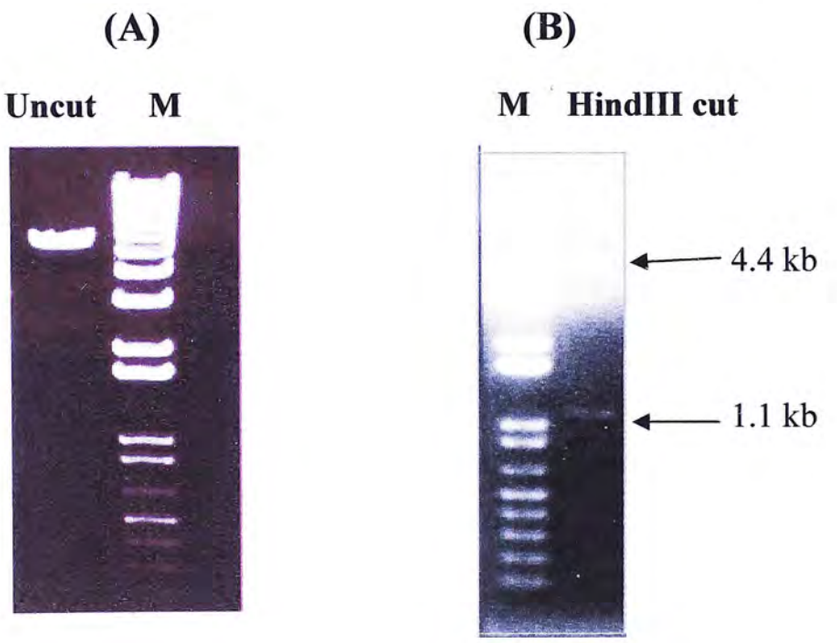
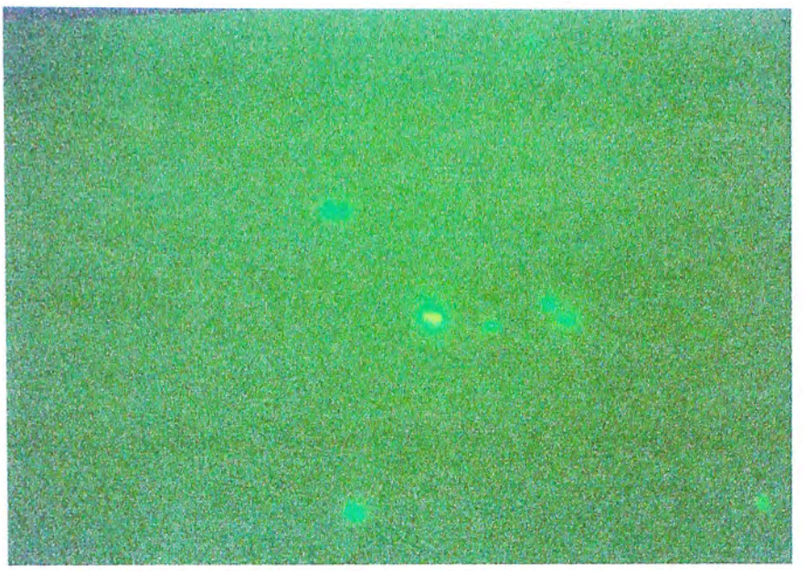


Fig. 41. The digestion mapping of pEGFP-Hyg with restriction enzyme – HindIII. (A), un-cut pEGFP-Hyg plasmid. (B), HindIII digested pEGFP-Hyg. DNA samples were loaded on 1% agarose gel and stained with EtBr. M, 1kb plus ladder.

(A)



(B)

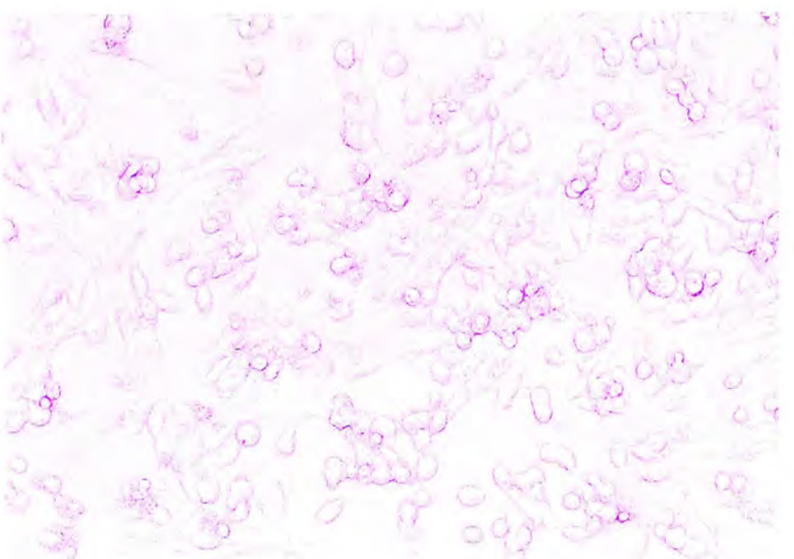


Fig. 42. Mouse 3T12 cells Transient transfected with pEGFP-Hyg. (A), image captured under the fluorescent microscope with 505-550-nm filter. (B), image captured under the fluorescent microscope without filter.

3.6.4.2 Construction of pEGFP-Hyg-Sj-PIG-N

pEGFP-HYG was used in the sub-cloning pEGFP-Hyg-Sj-PIG-N. The Sj-PIG-N gene is cut off from pTRE2 vector with BamHI and EcoRV and then ligated with BglII and SmaI digested pEGFP-N1 vector. Restriction enzyme EcoRV and XmnI were used for the restriction mapping test of pEGFP-N1-Sj-PIG-N (Fig. 43).

pEGFP-Hyg was used in the sub-cloning pEGFP-Hyg-Sj-PIG-N. 2.7μg of pEGFP-N1 was restriction digested with 10 units of BglII in 60μl final volume of 1x buffer H. The reaction mixture was incubated at 37°C for 3 hours, then purified by ethanol precipitation and finally resuspended in 10μl Milli-Q water. After that, 10μl of BglII digested pEGFP-Hyg was digested with 10 units of SmaI in 60μl final volume of 1x buffer J. The mixture was then incubated at 25°C for 3 hours. The digested products was purified by Qiagen gel extraction kit (referred to 2.2.3.4).

The Sj-PIG-N gene was prepared by digested with BamHI and EcoRV. 3μg of pTRE2-Sj-PIG-N was restriction digested with 12 units of EcoRV in 60μl final volume of 1x buffer D. After he reaction mixture was incubated at 37°C for 3 hours, it was purified by ethanol precipitation and finally resuspended in 30μl Milli-Q water. And then, 30μl of EcoRV digested pTRE2-Sj-PIG-N was digested with 10 units of BamHI in 60μl final volume of 1x buffer K. The reaction mixture was incubated at 30°C for 3 hours. The digested products was then purified (referred to 2.2.3.4).

6μl of Sj-PIG-N gene was ligated with 1μl of EcoRV and BamHI cut pEGFP-Hyg in a final volume of 10μl reaction mix. The ligation product was transformed into DH5α competent cells by heat shock transformation (referred to

2.2.3.7). The nucleotide sequence of cloning region was showed in (Fig. 44).

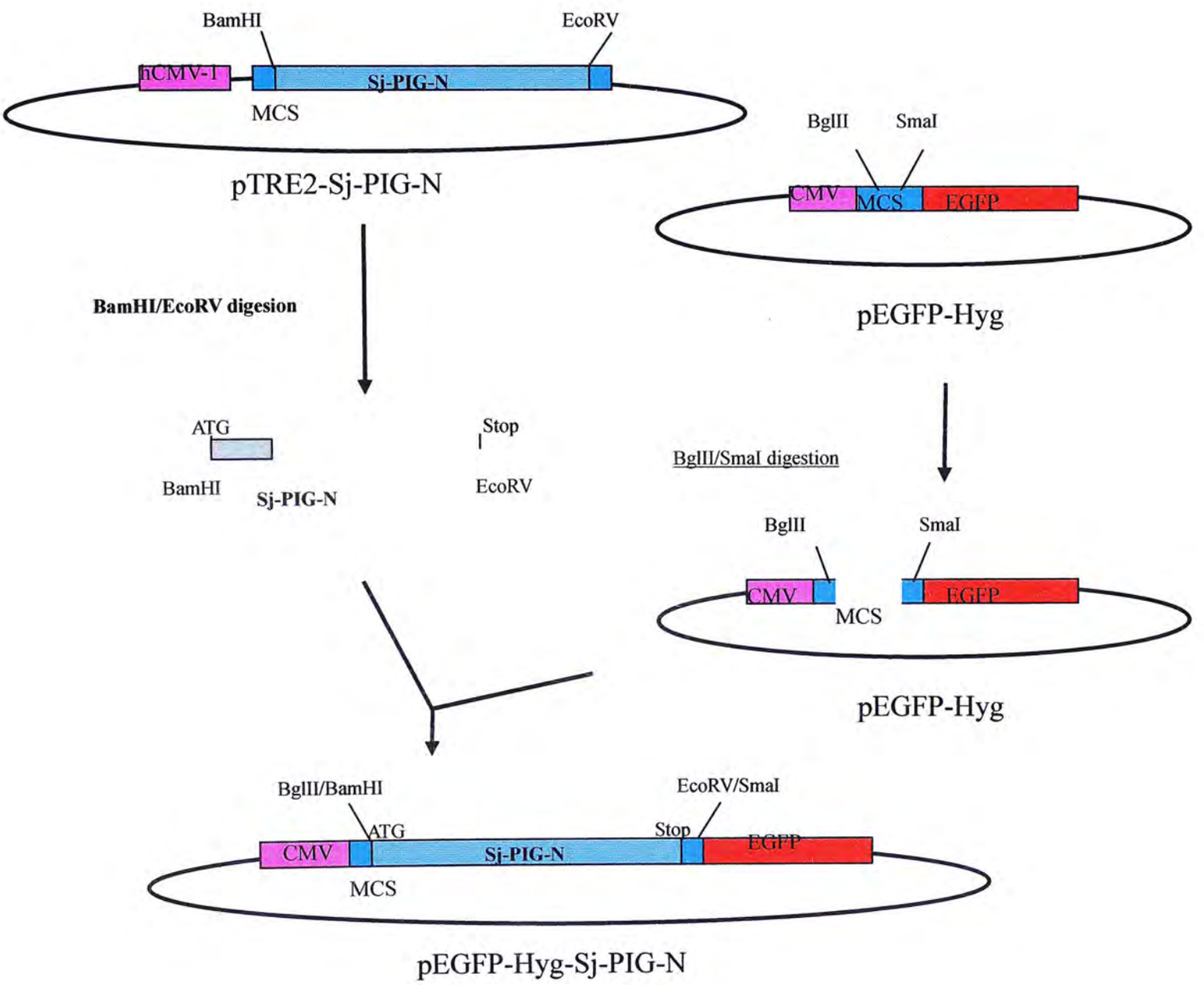


Fig. 43. Outline of construction of pEGFP-Hyg-Sj-PIG-N.

Fig. 44. Nucleotide sequences of cloning region of pEGFP-HygΔSj-PIG-N. The BamHI and EcoRV digested gene was cloned into pEGFP-Hyg vector that was prepared with BglII and SmaI digestion.

3.7 Molecular analysis of Sj-PIG-N protein

3.7.1 Functional analysis of Sj-PIG-N

3.7.1.1 FACS analysis of surface expression of GPI-anchored protein – Thy1

Function complementation test is the most directly method to identify a gene function. GPI anchor protein – Thy1 was used as a surface marker for the GPI anchor synthesis pathway in F9 cells (Hong, *et al.*, 1999). In order to identify the gene function of Sj-PIG-N, the plasmid DNA of pEGFP-Hyg-Sj-PIG-N was transfected into a mouse PIG-N double knock out cell line – F9 (PIG-N double knock out) cell line by DMRIE-C reagent (referred to 2.2.4.1). About $2x10^5$ mouse PIG-N double knockout F9 cells (PIG-N KO F9 cells) were seeded in a 60-nm tissue culture plate. When the cell density reached 50% confluent, a mixture containing 2μg of pEGFP-Hyg and 4μl of DMIRE-C reagent was transferred into the culture dish and incubated for 5 hours to allow the DNA enter the cells. After that, cells were selected with 150μg/ml hygromycine B. The cells were cultured in selection medium for 2 weeks, and the selection medium were changed every 2 days.

For the examination the surface expression of GPI-anchored proteins, cells were stained with anti-Thy1 (CD90.2) (referred to 2.2.5.1). They were analyzed in a FACScan (Becton Dickinson). The PIG-N KO F9 cells still expressed Thy1 on the cell surface. The expression of Thy1 on PIG-N KO F9 cells was lower than those on wild-type F9 cells. The expression of Thy1 on PIG-N KO F9 cells transfected with Sj-PIG-N was slightly higher than those on Pig-n KO cells (Fig. 45). Therefore, the cell surface expression of GPI-anchored protein – Thy1 can be restored by Sj-PIG-N.

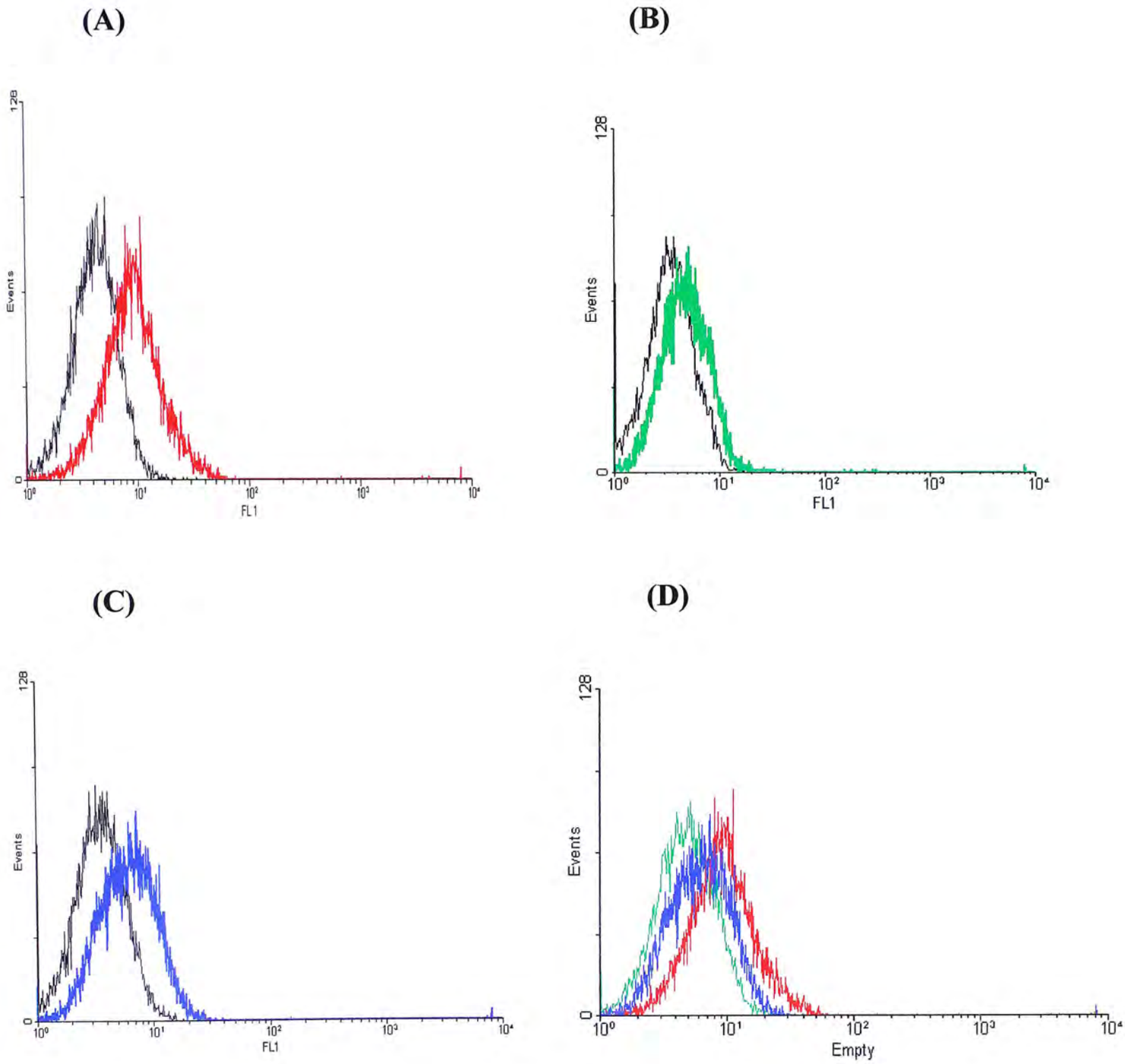


Fig. 45. Surface expression of Thy1 with FACS analysis. Blank cells (thin lines) and Cells stained with anti mouse thy1 antibody (bold lines) analyzed by flow cytometry. (A), wild-type F9 cells; (B), PIG-N KO F9 cells. (C), PIG-N KO F9 cells stably transfected with Sj-PIG-N. (D), comparison of Thy1 expression of F9 wild type cells, PIG-N KO F9 cells and PIG-N KO F9 cells transfected with Sj-PIG-N gene.

3.7.1.2 FACS analysis of surface expression of GPI-anchor protein - CD24

This part of experiment was collaborated with Professor Taroh Kinoshita, the Department of Immunoregulation, Resarch Institute for Microbial Diseases, Osaka University, Japan.

To express mouse Pig-n and Sj-PIG-N in Pig-n KO cells, their cDNAs were cloned into *Eco*RI and *Not*I sites of pMEbsd, pME bearing blasticidin resistance gene (Ohishi, *et al.*, 2000). Pig-n KO cells (1×10^7) were electroporated with 25 μg of linearized plasmids at 250 V and 500 μF using a Gene Pulser (Bio-Rad). Stable transfectants were obtained by selection in 4 μg/ml of blasticidin S for ten days (Ohishi, *et al.*, 2000). To examine the surface expression of GPI-anchored proteins, cells were stained with phycoerythrin-conjugated anti-CD24 M1/69 (BD Pharmingen). They were analyzed in a FACScan (Becton Dickinson).

Based on the FACS data, Sj-PIG-N can also restore the cell surface expression of GPI-anchored protein CD24 as well as the function of mouse Pig-n. The expression of CD24 on Pig-n KO F9 cells and Pig-n KO F9 cells transfected with FLAG-hGAA1, which was used as a negative control, were lower than those on wild-type F9 cells. The expression of CD24 on Pig-n KO F9 cells transfected with Sj-PIG-N, mouse Pig-n were higher than those on Pig-n KO cells transfected with FLAG-hGAA1 (Fig. 46).

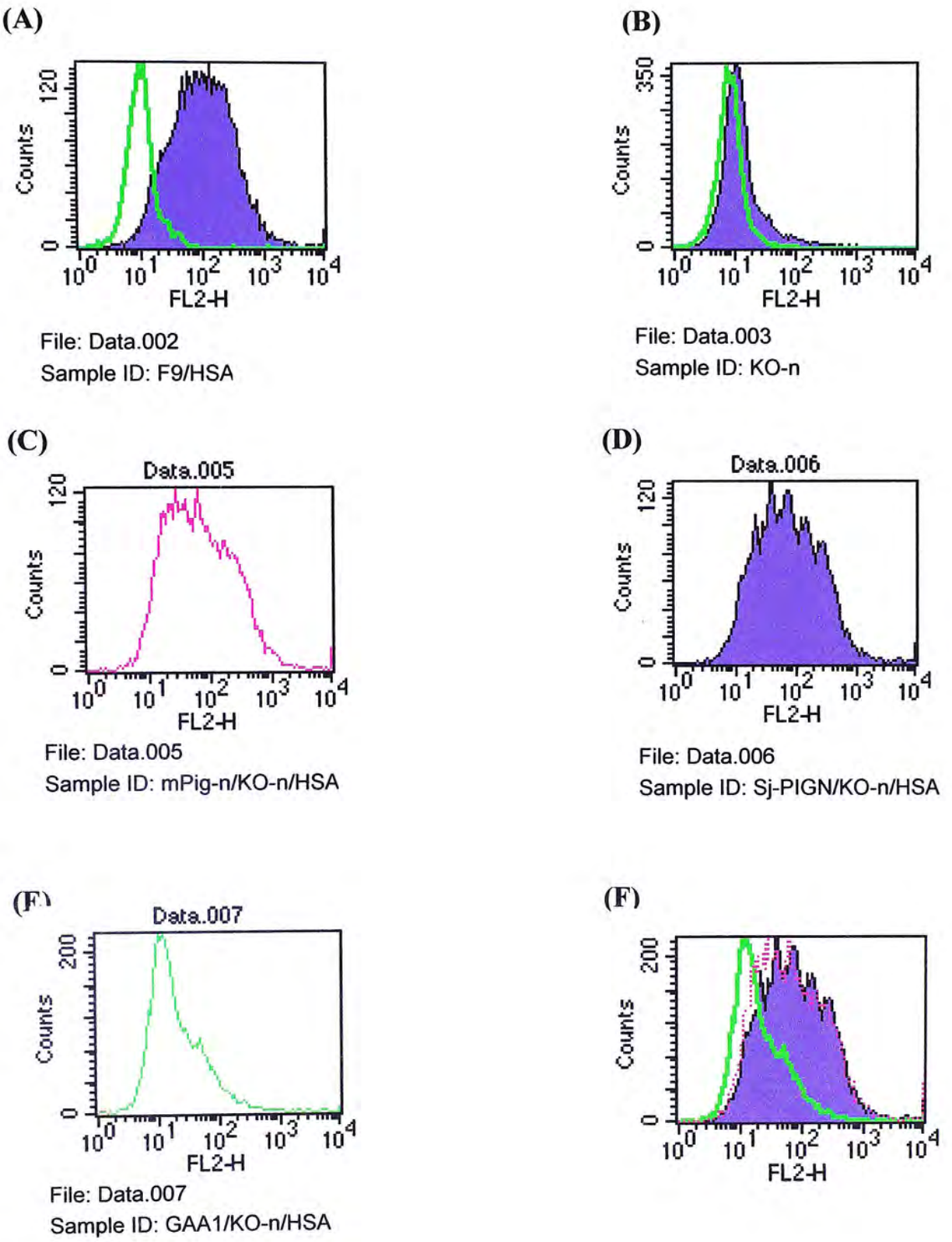


Fig. 46. Surface expression of CD24 with FACS analysis. Cells were stained with PE conjugated anti-CD24 antibody (bold lines) or control antibody (dotted lines) and analyzed by flow cytometry. (A), wild-type F9 cells; (B), Pig-n KO F9 cells. (C-E), Pig-n KO F9 cells stably transfected with mouse Pig-n (C), Sj PIG-N(D) or FLAG-hGAA1 as a negative control (E). (F), comparison of Pig-n KO F9 cells stably transfected with mouse Pig-n, Sj PIG-N and FLAG-hGAA1.

3.7.1.3 In Vitro Mannose Labeling of Microsomes and Characterization of Glycolipids

This part of experiment was collaborated with professor Taroh Kinoshita at Osaka University in Japan.

In order to test whether Sj-PIG-N can restore the ability to add EtNP to Man1 of GPI anchor, a synthetic substrate, GlcN-PI(C8) were used (referred to mannose assay). When the GlcN-PI(C8) was incubated with microsomes of F9 cells, Pig-n KOF9 cells, Pig-n KO F9 cells transfected with mouse Pig-n, Sj-PIG-N or FLAG-hGAA1 in the presence of palmitoyl-CoA and GDP-[^{3}H]mannose. The data clear showed that the Sj-PIG-N could restore the ability of transferring EtN-P to Man1 as well as the mouse PIG-N. For F9 cells, Pig-n KO cells transfected with mouse Pig-n, SjPIG-N, GlcN-PI(C8) was converted to radiolabeled Man-GlcN-(acyl)PI(C8), termed H2(C8), and further to a more polar product with addition of EtN-P to the mannose (marked as H5(C8)). However, for Pig-n KOF9 cells and Pig-n KO F9 cells transfected with FLAG-hGAA1, GlcN-PI(C8) was converted to H2(C8) and not to H5(C8) (Fig. 47).

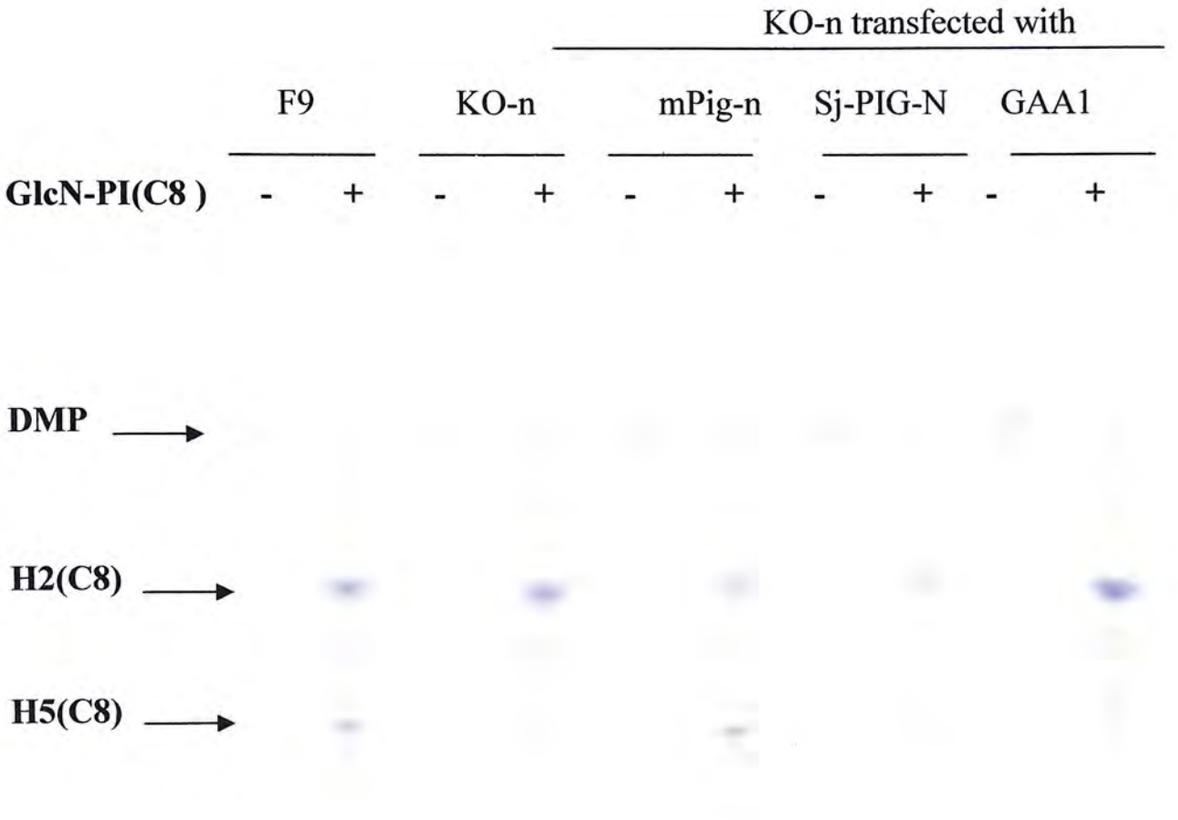


Fig. 47. Requirement of PIG-N for EtN-P modification of Man1 revealed by in vitro mannose labeling of microsomes. Microsomes (5×10^6 cell equivalents) from F9 (lanes 1, 2), Pig-n KOF9 cells (lanes 3, 4) and Pig-n KO cells transfected with mouse Pig-n (lanes 5, 6), SjPIG-N (lanes 7, 8) or FLAG-hGAA1 as a negative control (lanes 9, 10) were labeled with GDP-[^{3}H]mannose in the presence (lanes 2, 4, 6, 8, 10) or absence (lanes 1, 3, 5, 7, 9) of GlcN-PI(C8). DPM, dolichol –phosphate-mannose; H2(C8), Man-GlcN-acyl PI(C8); H5(C8), EtNP-Man-GlcN-acyl PI(C8).

3.7.2 Localization of Sj-PIG-N with immunofluorescent staining

This part of experiment was collaborated with professor N. K. Mak at the Hong Kong Baptist University.

To identify the location of Sj-PIG-N protein in mammalian cells, the anti-mouse KDEL antibodies or *S. japonicum* infected rabbit serum (SJR) were used to stain the PIG-N KO F9 cells transfected with SJ-PIG-N (constructed in 3.5.4.2) and the PIG-N KO F9 cells. The KDEL is a marker protein of ER (Kaisto and Metsikko, 2003). The stained cells were analyzed under confocal laser scanning microscope (Zeiss, Model LSM510) (referred to protocol). The expressions of KDEL proteins were detected with a 650-nm long-pass filter and the expression of SJ-PIG-N was detected under a 505-550-nm band-pass filter. Under the confocal microscope, the KDEL proteins were stained as red color; the Sj-PIG-N proteins were stained as green color. After combined the two images, the pink color clearly indicated that the Sj-PIG-N located in ER as well as KDEL proteins (Fig. 48).

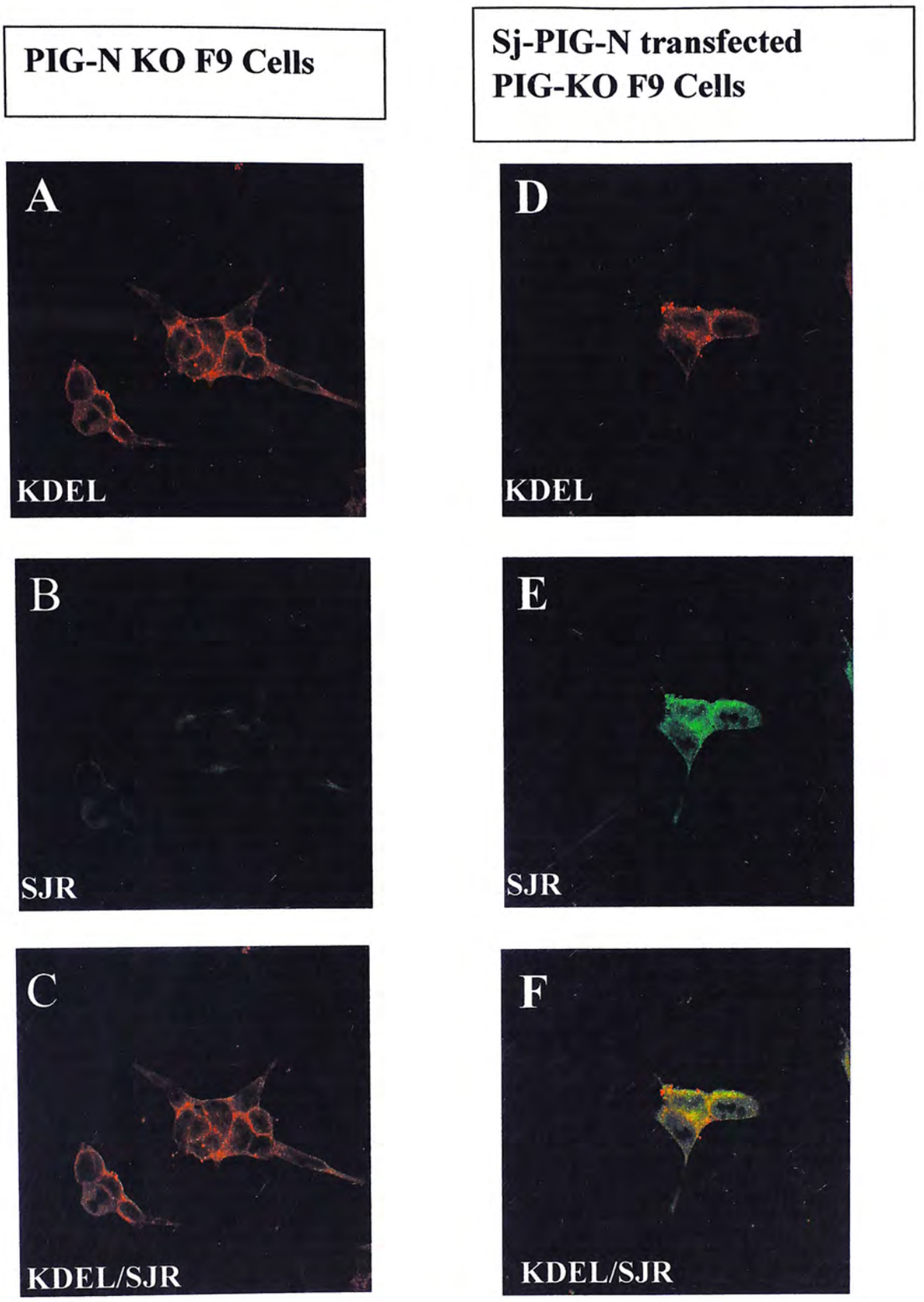


Fig. 48. Sj-PIG-N localization in PIG-N KO F9 cells transfected with Sj-PIG-N. Pig-n KO F9 cells and PIG-N KO F9 cells stable transfected with Sj-PIG-N were stained with the anti-mouse KDEL antibodies (KDEL) (A) and (D); *S. japonicum* infected rabbit serum (SJR), (B) and (E); The image of PIG-N KO F9 cells stained with anti-mouse KDEL antibodies or *S. japonicum* infected rabbit serum were combined, (C); The image of PIG-N KO F9 cells transfected with Sj-PIG-N stained with anti-mouse KDEL antibodies or *S. japonicum* infected rabbit serum were combined, (F).

Chapter Four Discussion

4.1 *S. japonicum* cercaria EST analysis

Schistosomiasis is the second prevalent parasitic disease in the world. It is epidemic in 74 countries and territories with over 200 millions people affected. Chemotherapy is the only way to treat the disease. Although it is effective method, it cannot prevent the re-infection. For the development of new treatment and effective prevention approaches, it is an urgent task for scientist to expand our knowledge of schistosome in its genetic aspect. Genetic study of schistosome is very important for us to understand its biology, mechanism of immune evasion.

After the establishment of Schsitosma Genome Project (SGP) in 1992, the genetic analysis of *S. japonicum* has undergone a rapid expansion. Until 2001, there are approximately 2000 Expressed sequence tags (ESTs) of *S. japonicum* have been deposited in databases (Fung *et al.*, 2002). ESTs constitute a rapid and informative strategy for studying gene-expression profiles of specific stages of schistosome.

Attenuated cercaria is a very effective live vaccine to prevent the parasite infection in *S. japonicum* (McManus, 1999). As it can evoke effective protective immunity against challenge infection in animal model (Moloney *et al.*, 1985; Bickle *et al.*, 1985), it is believed that cercaria stage specific genes play a role in the case. Therefore, the main attentions in schistosome genetic research are focused on genes or their gene products are present in different expression levels in determined stages and stage specific genes (Hawn and Strand, 1994; Xu *et al.*, 1989; Smith *et al.*, 1992;

Pierrot *et al.*, 1996).

In my studies, the *S. japonicum* cercaria cDNA library was used to screening genes expressed in cercaria stage. The cDNA library was constructed by a visitor scientist - Dr. X.G. Chan in our library in 2000. Based on the report of cDNA library (Fung, WHO/TDR/DIF project report, 2000), the percentage of recombinant clones is 94.4%. However, in my experiment, eleven phage plaques were randomly selected and sequenced. Only seven of them carrying an insert, the other four are failed in cloning during the library construction. The percentage of recombination clones is much lower than the report.

In order to predict the putative gene function of those clones, the sequence of these clones were applied in BlastX search. For the seven clones carrying inserts, only one gene, clone P06, which has been identified and reported in gene bank. The clone P06 is homologous to *Schistosoma japonicum* calcium-binding protein (Peng *et al.*, 2003) and their identity is 100% (Fig. 14).

The clone P02 showed a very high homology to *S. mansoni* thioredoxin glutathione reductase with 97% identity. In *S. mansoni*, thioredoxin glutathione reductase plays an important role in the thiol redox system for detoxification. Residing in the hepatic portal system, adult schistosome is exposed to reactive oxygen compounds. The function of this enzyme is to minimize the oxidative stress (Alger and Williams, 2002)

For the clone P08, it is a homologous of phosphatidylinositol glycan class N (PIG-N). The function of PIG-N is to transfer an EtN-P to the first mannose of

glycosylphosphatidylinositols (GPI) anchor during the GPI anchor synthesis (Gaynor *et al*,. 1999; Hong *et al*., 1999).

The clone P05 showed a relatively high homology to translation elongation factor 1-gamma (EF-1γ) (Sanders *et al*., 1992) with 51% identity. Elongation factors are encoded, in most cases, by housekeeping genes in eukaryotes and prokaryotes. Although, their basic function is involved in protein synthesis, they could express a variety of other important and some times surprising biological properties. For example, the *T. cruzi* EF-1γ could direct components of the protein synthesis apparatus towards the three-dimensional cytoplasmic structure of the cell (Janssen and Möller, 1988). Over expression of EF-1γ in *T. bruzi* can enhance the drug resistance ability of the parasite (Billaut-Mulot *et al*., 1997; Ouaissi, 2003). In addition, *EF-1γ* could be a useful marker as *ras* gene in non-familial adenomatous polyposis (Lew *et al*., 1992) and provide preoperative useful information for the prediction of tumor aggression (Mimori *et al*., 1996).

For the clone P9, it has 66% identity to human elongation factor 2 (Hanes *et al*., 1992). The activity of eukaryotic elongation factor-2 (eEF2), which is critically important for the elongation of mRNA translation, is regulated by phosphorylation (Nairn *et al*., 1985; Kigoshi *et al*., 1989; Carlberg *et al*., 1990).

The clone P07 is a homologous of 40S ribosomal protein S27, and has 63% identity to the protein (Fernandez *et al*., 1993; Tsui *et al*., 1996). Although, the clone N01 showed a 32% identity to human envelope glycoprotein, its E value was too high and the score value was too low. Therefore, the clone N01 might be a novel gene in *S. japonicum*.

Although, there are only seven genes that have been identified and one of it is a known gene, some of them are valuable for a further study. The clone P02, a homologous of *S. mansoni* thioredoxin glutathione reductase, may play an important role in detoxification mechanism that is related to the survival of parasite within its host. The clone P05, a homologous of translation elongation factor 1-gamma, may be important in drug resistance in schistosome.

Our study was focused on the PIG-N homologous gene – clone P08, which is named as Sj-PIG-N. In parasites, the GPI anchor proteins play important roles in immune evasion. The typical example is the VSG in *T. brucei*. In schistosome, the GPI anchored proteins are important in antigen variation (Sauma *et al.*, 1991; Hawn and Strand, 1992); host lipoprotein and glucose uptaking (Goldring *et al.*, 1976; Sher *et al.*, 1978; Ramalho-Pinto *et al.*, 1978; Conford *et al.*, 1983; Bryant, 1993; Camacho and Agnew, 1995); and mimic host cells (Tempone *et al.*, 1997; Xu and Caulfield, 1992). Moreover, until now, no GPI anchor synthesis protein has been reported. The clone P8 possibly the first gene cloned for GPI anchor synthesis in schistosome.

4.2 Structure analysis of Sj-Pig-N gene

The clone P08 only contained partial sequence of the Sj-PIG-N gene. For the further functional and structural analysis of Sj-PIG-N gene, it is necessary to obtain the full-length sequence of the gene. For obtaining of the 5' & 3' cDNA ends of the Sj-PIG-N gene, SMART^{TM} RACE cDNA ampification kit (Clontech K1811-1) was used. Based on the 3' and 5' cDNA ends sequence, the gene specific primers MF834 and MF838 for amplification of full length gene were designed. A gene around 3000

base pairs were amplified with MF834 and MF838 by RT-PCR (Fig. 22). Successful amplification of full length Sj-PIG-N gene by a pair of gene specific primers is a direct evidence of presence of the gene in the parasite. Although the full length gene can be also obtained from construction of several parts of the gene with RT-PCR and ligation, we cannot prove that each part of Sj-PIG-N gene is amplified from Sj-PIG-N gene, Sj-PIG-N gene families or other genes that share identical sequence at some regions.

The direct sequence method was used in the obtaining Sj-PIG-N sequence. In the Polymerase Chain Reaction (PCR), Taq DNA polymerase creates one incorrect base in every 10,000 base pairs that leads to a high mutation rate. However, direct sequencing of PCR product can eliminate the problem. According to the direct sequencing result, the coding region of Sj-PIG-N gene contains 2964 base pairs. After translation, Sj-PIG-N protein has 987 amino acids.

Although the sequence of clone P08 were homologous to that of PIG proteins in BlastX search, the sequence of P08 was just a partial sequence of Sj-PIG-N. It is necessary to perform a more precise BlastP search using the translated Sj-PIG-N protein sequence to confirm BlastX search results of the P08. As we predicted, the Sj-PIG-N proteins showed very homology to Mcd4 proteins and PIG-N proteins of human, mouse, and *C. elegan*. In the BlastP search, the SJ-PIG-N proteins were detected to contain putative conserved domains of AP superfamily, phosphatiylinositolglycan class N (PIG-N), metalloenzyme superfamily, phosphodiesterases / nucleotide pyrophosphatase, phosphoglyceromutase and arylsulfatase A related enzymes (Fig. 24). Except the homologous score of putative domain of arylsulfatase A related enzymes is relatively lower, all of the others are quit

high in the score. The putative PIG-N domain gives us more confidence that the SJ-PIG-N has the similar function of other PIG-N proteins. In addition, it is interesting, that all of the PIG-N proteins shared three conserved motifs of known mammalian phosphodiesterases and nucleotide pryphophatases (Gaynor *et al.*, 1999). The function of PIG-N proteins is to add EtN-P to the first mannose of GPI main core. The enzyme may cleave a nucleotide bound EtN-P precursor (i.e. CDP-ethanolamine) to generate a EtN-P moiety with its nucleotide pyrophosphatases activity, and then add the EtN-P to the mannose by creating a phosphodiester bond with its phosphodiesterase acitivity. The three conserved motifs were also detected in Sj-PIG-N protein (Fig. 25) in the CLUSTALW Multiple Sequence Alignment.

For the understanding more in the feature of Sj-PIG-N protein, it was analyzed with a signal peptide cleavage sites prediction program SignalP V2.0 (Nielsen, *et al.*, 1997), a transmembrane domain prediction program TopPred2 (Von Heijne, 1992; Claros and Von Heijne, 1994) and a program for glycosylation sites prediction - NetNGlyc 1.0 (Gupta *et al.*, 2002). Based on the predicted results, there are sixteen predicted trans-membrane domains (TMDs) (Fig. 29) and four N-linked glycosylation sites in Sj-PIG-N protein (Fig. 30). In addition, the Sj-PIG-N protein contains a 21 amino acids signal peptide (Fig. 28). After combined the CLUSTALW Multiple Sequence Alignment and the topology prediction results, we found that the region of highest homology is within the ER lumen domains. It is well known that each of PIG-N proteins are transmembrane proteins, usually multimembrane spanning. The topology of transmembrane domains of Yeast PIG-N protein, MCD4, is quit similar to that of Sj-PIG-N, except there are only 14 hydrophobic domains in MCD4 (Gaynor *et al.*, 1999). Like other PIG-N proteins, Sj-PIG-N also has a C-terminal KKXX motif, which mediates Golgi to ER retrieval of membrane proteins by directing their

packaging into retrograde COPI-coated vesicles (Letourneur *et al.*, 1994).

The original motive of screening the *S. japonicum* cercaria stage cDNA library is to identify certain cercaria stage specific genes or genes that highly expressed at the stage. Therefore, to test whether the Sj-PIG-N gene is cercaria stage specific or not, RT-PCR was applied to amplified the Sj-PIG-N gene fragment in the RNAs of adult worms and cercaria. In this experiment, a pair of gene specific primers – MF802 and MF804 was used and the predicted gene product was 860 base pairs. The result indicated that the Sj-PIG-N gene expressed in both stages of adult worms and cercaria and the expression level in the two stages might be similar. As there is no housekeeping gene has been identified in *S. japonicum*, it is difficult to normalize the RT reaction. Due to the limited amount of total RNA of both stages, it is impossible to perform a Northern Blot analysis for the Sj-PIG-N gene. As for each lane of sample in Northern Blot analysis, 10μg of total RNA was required (Williamson *et al.*, 1989). Moreover, as we do not have the RNA from other stages of *S. japonicum*, for example, eggs, we cannot identify whether the gene express in these stages. Therefore, we can only prove that the Sj-PIG-N gene express in both adult worms stage and cercaria stage.

4.3 Molecular analysis of Sj-PIG-N

In the main core structures of both mammalian cells and yeast cells, Man1 in GPI is modified by addition of a side branching EtN-P with PIG-N enzymes. Unlike the core structure of GPI anchor, such as the core mannose residues, the side-branching EtN-P moieties do not act as building blocks on the which the rest of the anchor that must be constructed. Therefore, failure of adding the side chain may

not completely hinder the synthesis of GPI anchor synthesis; it may cause the precursor GPI anchor to form a less efficient substrate, lead to accumulation of a series of aberrant GPI precursor that all lack EtN-P substitute.

In mammalian cells, the modification Man1 with EtN-P is not very essential because the surface expression of GPI anchored proteins was only partially affected by disruption of PIG-N. Thy1, one of GPI anchored proteins, was used as a marker of successful GPI anchor synthesis. In mouse F9 cell line, double knock PIG-N gene can significantly decrease the Thy1 expression on cell surface, but cannot completely shut down the its surface expression (Hong *et al.*, 1999). However, the modification is very important for the cell growth in yeast. In *Saccharomyces cerevisae*, the mutated MCD4 gene does not generate functional GPI anchor at nonpermissive temperature that cause the cells cannot form buds (Gaynor *et al.*, 1999).

The Blast search result and the topology prediction of Sj-PIG-N clearly indicate it is a homologous of phosphatiylinositolglycan class N proteins and it possibly plays a similar role in GPI anchor synthesis as other PIG-N proteins do. Therefore, Functional test is a must for identify the role of Sj-PIG-N in the cells. In our study, the ability of restoring membrane protein, Thy1, was demonstrated by FACS analysis (Fig. 45). But the differences of membrane Thy1 expression between the PIG-N KO F9 cells and F9 cells, or PIG-N KO F9 cells and PIG-N KO F9 cells stable transfected with Sj-PIG-N only showed a little significance. Both the PIG-N KO F9 cells and F9 cells are the gift from Professor Kinoshita, and in his publication, the difference of membrane Thy1 expression between the PIG-N KO F9 cells and F9 cells was about 10 fold. For our result, it may be due to the clonal variation of Thy1 expression in F9 cells (personal communication with Professor Kinoshita). For confirmation of the

function of Sj-PIG-N, the functional test of the gene was also performed by the research team of Professor Kinoshia. In their study, they found the Sj-PIG-N can restore the surface expression of GPI anchor protein, CD24 in PIG-N KO F9 cells stable transfected with Sj-PIG-N gene (Fig. 46). Their results clearly indicate that he difference of membrane Thy1 expression between the PIG-N KO F9 cells and PIG-N KO F9 cells stable transfected with Sj-PIG-N was about 10 fold.

In addition, the research team of Professor Kinoshita also performed a biochemical functional test for Sj-PIG-N. *In vitro* mannose labeling of microsomes demonstrated that Sj-PIG-N gene can of restore the ability of EtN-P modification of Man1 (Fig. 47). As incubation of GlcN-PI with mammalian cell membranes in the presence of palymitoyl-CoA and GDP-mannose resulted in inositol-acylation and mannosylation of the GlcN, generate Man-GlcN-(acyl)PI (Doerrler *et al.*, 1996). After that, the Man-GlcN-(acyl)PI will convert into (EtN-P)-Man-GlcN-(acyl)PI (Hong *et al.*, 1999). In the in vitro mannose labeling assay, PIG-N KO cells and the negative control, FLAG-hGAA1 transfected PIG-N KO F9 cells, did not form EtN-P-Man-GlcN-(acyl)PI structure and the mannose labeling products were accumulated at Man-GlcN-(acyl)PI stage. Sj-PIG-N transfected PIG-N KO F9 cells showed a similar result with mouse PIG-N transfected PIG-N KO F9 cells. Both of the two cells showed the accumulation of (EtN-P)-Man-GlcN-(acyl)PI. Therefore, it is reasonable to believe that Sj-PIG-N has the function of adding EtN-P to the Man1 of GPI core structure.

The PIG-N KO F9 cells stable transfected with Sj-PIG-N gene stained with *S. japonicum* infected rabbit serum and the anti-mouse KDEL antibodies under the analysis of confocal laser scanning microscope indicating that Sj-PIG-N located on

the ER lumen membrane (Fig. 48). KDEL is a typical ER membrane protein using as a marker for identification of ER proteins (Kaisto and Metsikko, 2003).

4.4 Further study

In protozoa parasite, the GPI anchored proteins play an important role in the survival of these organisms. For *T. brucei*, Tehre are about 5 x 10^6 PI anchored variant surface glycoprotein (VSG) dimers that protect the parasite from the host immune attack (Cross, 1996; Mehlert *et al.*, 1998). The specific inhibitors of parasite GPI biosynthesis pathways are believed to be useful for the development of anti-parasitic agents.

From mammalian to protozoa, the biosynthesis pathway of GPI anchor is very similar. In all the cases, GPI anchor synthesis involves the addition of GlcNAc to phosphatidylinositol (PI) to generate GlcNAc-PI, which is de-N-acetylated to form GlcN-PI (Doering *et al.*, 1989; Hirose *et al.*, 1991; Stevens, 1993; Milne *et al.*, 1994; Nakamura *et al.*, 1997). N-ethyl-maleimide (NEM) specific inhibits α1-6 GicNAc transferase, which is the first enzyme of GPI anchor biosynthesis in trypanosome cell-free system and HeLa cell-free system (Milne *et al.*, 1992). However, the GlcN-(2-O-hexadecyl)PI can only inhibit the α1-6 GicNAc transferase in *T. brucei*, but cannot inhibit the GPI synthesis of mammalian cells (Smith *et al.*, 1999). For both trypanosome and mammalian cells, the de-N-acetylase, which is involved in GlcNAc-PI de-N-acetylation, can be inhibited by D-GlcNMe$_2$α1-6D-myo-inositol-1-HPO$_4$-sn-1,2-dipalmitoylglycerol (GlcNMe$_2$-PI) or 2-deoxy-2ureido-D-Glcα1-6D-myo-inositol-1-HPO$_4$-sn-1,2-dipalmitoylglycerol (GlcNCONH$_2$-PI).

In the synthesis of GPI main core structure, GPI mannosyltransferases are responsible for the addition of mannose residues on the GPI precursor. The activity of the enzymes can be inhibited by D-Glcα1-6D-myo-inositol-1-HPO_4-sn -1,2-dipalmitoylglycerol and its diastereoisomer, D-Glcα1-6L-myo-inositol-1-HPO_4-sn -1,2-dipalmitoylglycerol in T. brucei cell-free system (Smith *et al.*, 2000). As both of them has no effect on GPI biosynthesis in human HeLa cell-free system, indicating that these molecules are parasite-specific inhibitors.

A terpenoid lactone, YW 3548, was firstly identified as an inhibitor for blocking the addition of the third mannose to the intermediate structure Man-Man-Glc-(acyl)PI in both mammalian cells and yeast cells and have no effect on the GPI synthesis of trypanosome (Sutterline *et al.*, 1997). Later, it was proved that YW 3548 also target on the enzyme of mouse PIG-N and yeast MCD4 (Hong *et al.*, 1999) in the GPI anchor synthesis. Except YW 3548, a metalloportease inhibitor – 1,10-phenanthroline (PNT) can also inhibit the activity of mouse PIG-N. PNT actually inhibit all of the three phosphoethanolamine transferases in mammalian cell model (Mann and Sevlever, 2001). The PNT also can inhibit the GPI anchor synthesis of yeast, but has no effect on the synthesis of GPI anchor in trypanosome (Sevlever, 2001).

As the existence of trypanosome specific inhibitors (e.g. D-Glcα1-6L-myo-inositol-1-HPO_4-sn-1,2-dipalmitoylglycerol) and mammalian specific inhibitors for GPI anchor synthesis. It is reasonable to believe that the schistosome specific inhibitor for their GPI anchor synthesis pathway could be developed. In schistosome, GPI anchored proteins are involved in the immune evading mechanism, lipoproteins uptaking, and glucose absorption those are very important for the parasite survival inside host. Inhibition of the GPI anchor synthesis

will disrupt the immune protection and physiological function of the parasite and lead to parasite death.

Sj-PIG-N is the first PIG-N homologue discovered in parasite and we do not know whether it is essential in the PGI anchor synthesis in schistosome cells, although the MCD4 is essential for generation of a sufficient amount of functional GPI in yeast. It is still valuable to screen the Sj-PIG-N specific inhibitors. The PIG-N KO F9 cell line transfected with Sj-PIG-N gene will be a good cell model to test the effect of potential Sj-PIG-N inhibitor. Screening of Sj-PIG-N specific inhibitor is a complex and long-term task. At the beginning, we should test the effect of mouse PIG-N inhibitors – YW 3548 or PNT on Sj-PIG-N activity that may provide valuable hinds for further drug screening.

4.5 Conclusion

In these studies, Sj-PIG-N, which is a *S. japonicum* homologoue of yeast MCD4, and mouse PIG-N, was characterized. We also demonstrated its function that it is involved in the transferring of EtN-P to the first mannose in the GPI main core structure. The full-length cDNA of Sj-PIG-N allows us to construct a cell model to screen for the *S. japonicum* specific PIG-N inhibitors and to evaluate the potential of Sj-PIG-N as a vaccine against *S. japonicum*.

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